EFFECT OF TETRACYCLINES ON OSTEOPENIC- AND OSTEOPOROTIC-DERIVED BONE-MARROW OSTEOBLASTIC CELL CULTURES

Joana Pereira de Pinho

Dissertação para obtenção do grau de Mestre em Engenharia Biomédica

Faculdade de Engenharia, Universidade do Porto

Porto, julho de 2013

EFFECT OF TETRACYCLINES ON OSTEOPENIC- AND OSTEOPOROTIC-DERIVED BONE-MARROW OSTEOBLASTIC CELL CULTURES

Orientadores

Professor Doutor Pedro Sousa Gomes

Faculdade de Medicina Dentária da Universidade do Porto

Professora Doutora Maria Helena Raposo Fernandes

Faculdade de Medicina Dentária da Universidade do Porto

Faculdade de Engenharia, Universidade do Porto

Porto, julho de 2013

INDEX

AGRADECIMENTOS

Ao Professor Doutor Pedro Sousa Gomes e à Professora Doutora Maria Helena Fernandes, agradeço o apoio e incentivo constantes, a disponibilidade, os ensinamentos transmitidos no último ano e meio, e a supervisão do trabalho de investigação que levou à realização desta dissertação.

À Dra. Mónica Garcia e ao José Carlos do Laboratório de Farmacologia e Biocompatibilidade Celular da Faculdade de Medicina Dentária, Universidade do Porto, agradeço a motivação, paciência e ajuda nos procedimentos laboratoriais.

Aos meus amigos, em especial à Sofia Ribeiro, agradeço a apoio e amizade durante a elaboração desta dissertação.

Aos meus pais e irmão, agradeço a motivação e amor incondicionais, não só durante esta fase, como ao longo de todos os momentos da minha vida.

ABSTRACT

Osteoporosis is a pathological condition characterized by a reduction in bone mass and modifications in bone microarchitecture, resulting in increased bone fragility and fracture risk. These fractures are widely recognized as a major health problem in the elderly population. Preliminary data reports that the bone regeneration process, in osteoporotic conditions, may be somewhat limited mainly due to a decrease of new bone formation. Nowadays, current bone tissue engineering strategies require biomodulators to achieve a successful regeneration of the bone tissue. Tetracyclines are suitable candidates for bone regenerative strategies because they have high affinity to mineralized tissues and an antibacterial action.

This experimental work aims to establish two animal models, representative of the human osteopenic and osteoporotic conditions, and to assess the *in vitro* effect of doxycycline in the cellular behavior of grown osteoblastic cultures established from osteopenic and osteoporotic conditions. Briefly, rat bone marrow-derived osteoblastic cultures were established from three different experimental conditions – sham, osteopenic and osteoporotic animals – and grown in the presence of doxycycline, in order to depict its effect in cell proliferation, metabolic and functional activities.

Cell cultures established from the sham group shown an increased cell proliferation in the presence of doxycycline. Doxycycline was also found to enhance metabolic activity and ALP expression of the established cultures. Cultures established from osteopenic animals revealed an increased cell proliferation that was stimulated in the presence of doxycycline. The expression of ALP was also improved with addition of doxycycline, being maintained the osteoblastic phenotype during the time of culture. In cultures established from osteoporotic animals, doxycycline delayed the cell proliferation and metabolic activity, so cell confluence was reached later. On the other hand, the ALP activity was enhanced by the addition of doxycycline. In cultures established from the three metabolic conditions, an enhanced expression of osteocalcin and osteopontin were also verified in the presence of doxycycline, as well as a reduction in some adipogenic markers.

The results present in this study revealed that doxycycline can improve the osteogenic potential of osteoblast precursor cells in osteopenic and osteoporotic conditions. Therefore, these antimicrobial drugs can be useful in tissue engineering and have a potential biomedical application, being used in the management of osteoporosis and related conditions.

RESUMO

A osteoporose é uma condição patológica caracterizada pela diminuição da massa óssea e alteração na arquitetura microscópica do osso, tendo como resultado um aumento da fragilidade óssea e do risco de fractura. Estas fracturas são reconhecidas como um importante problema de saúde na população mais idosa. Segundo dados prévios, o processo de regeneração óssea, em condições de osteoporose, pode ser limitado devido, principalmente, a uma diminuição na formação de novo osso. Atualmente, estratégias de engenharia de tecidos assentam na utilização de biomoduladores para que a regeneração do tecido ósseo seja alcançada com sucesso. As tetraciclinas são candidatos apropriados para estratégias de regeneração óssea devido à sua elevada afinidade para tecidos mineralizados e à sua ação antibacteriana.

Este trabalho experimental tem como objetivo o estabelecimento de dois modelos animais representativos das condições de osteopenia e osteoporose humanas, e a avaliação *in vitro* do efeito da doxiciclina no metabolismo e atividade funcional de populações osteoblásticas derivadas de condições osteopénicas e osteoporóticas. Foram estabelecidas culturas osteoblásticas derivadas de medula óssea de rato em três condições experimentais diferentes – sham, osteopénica e osteoporótica – e foi avaliado o efeito da doxiciclina na proliferação e atividade funcional das referidas culturas.

As culturas celulares estabelecidas a partir do grupo sham demonstraram um aumento na proliferação celular na presença de doxiciclina. A doxiciclina também revelou um aumento da atividade metabólica e da expressão de ALP nestas culturas. As culturas estabelecidas a partir de animais osteopénicos revelaram um aumento na proliferação celular que foi estimulada na presença de doxiciclina. A expressão de ALP também aumentou com a adição de doxiciclina, sendo assim mantido o fenótipo osteoblástico ao longo do tempo de cultura. Nas culturas estabelecidas a partir de animais osteoporóticos, a doxiciclina atrasou a proliferação celular e a atividade metabólica, levando a que a confluência celular fosse alcançada mais tarde. Por outro lado, a atividade da ALP foi estimulada com a adição de doxiciclina. Nas culturas estabelecidas a partir destas três condições metabólicas, verificou-se um aumento na expressão de osteocalcina e osteopontina na presença de doxiciclina, tal como uma redução de alguns marcadores adipogénicos.

Os resultados apresentados neste estudo revelaram que a doxiciclina pode melhorar o potencial osteogénico de células precursoras de osteoblastos em condições de osteopenia e osteoporose. Deste modo, este medicamente antimicrobial pode ser útil na engenharia de

tecidos e tem potenciais aplicações biomédicas, podendo ser utilizado no tratamento da osteoporose e condições relacionadas.

FIGURE INDEX

GRAPHIC INDEX

ABREVIATION LIST

AA – Ascorbic Acid

ALP – Alkaline Phosphatase

AMV-RT – Avian Myeloblastosis Virus RT

BMD – Bone Mineral Density

BMP – Bone Morphogenetic Protein

BMU – Bone Multicellular Unit

BV/TV – Bone Volume/Total Volume

Cbfa1 – Core-binding Factor α1

CLSM – Confocal Laser Scanning Microscopy

CMT – Chemically Modified Tetracyclines

CNS – Central Nervous System

CO_2 – Carbon Dioxid

CT – Computed Tomography

DNA – Deoxyribonucleic Acid

dNTP – Deoxynucleoside Triphosphate

DTT – Dithiothreitol

DXA – Dual-Energy X-ray Absorptiometry

D1 – Doxycycline

ECM – Extracellular Matrix

FBS – Fetal Bovine Serum

FUNGI – Fungizone

GH – Growth Hormone

HA – Hydroxyapatite

IGF-I – Insulin-like Growth Factor-I

IGF-II – Insulin-like Growth Factor-II

IL-1 – Interleukin-1

IP – Intraperitoneal

MMP – Matrix Metalloproteinase

MSC – Mesenchymal Stem Cell

NaOH – Sodium Hydroxid

OC – Osteocalcin

OPG – Osteoprotegerin

OPN – Osteopontin

Osx – Osterix

PDGF – Platelet-derived Growth Factor

PEST – Penicillin-streptomycin

pNPP – p-nitrophenylphosphate disodium

PTH – Parathyroid Hormone

QUS – Quantitative Ultrasound

RANK – Receptor Activator of Nuclear Factor κB

RANKL – RANK Ligand

Tb N – Trabecular Number

Tb Sp – Trabecular Separation

Tb Th – Trabecular Thickness

TGF-β – Transforming Growth Factor-β

TNF – Tumor Necrosis Factor

TRAPase – Tartrate-resistant Acid Phosphatase

VOI – Volume-Of-Interest

WHO – World Health Organization

α-MEM – α-Minimal Essential Medium

I. LITERATURE OVERVIEW

BONE TISSUE

BONE STRUCTURE

Bone is a connective tissue considerable complex and important since it is responsible for strength and stiffness of the skeleton, ensuring the protection of soft tissues and organs (1). Bone is constantly remodeling (bone resorption and bone formation) (2) and has two main components: the extracellular bone matrix and bone cells (3).

Due to its unique characteristics, its material composition and structural properties (4), bone tissue has various functions of extreme importance in the human body such as:

- Movement and locomotion (3);
- Support – shape the skeleton that serves to support muscle insertion (3);
- Protection – protects internal tissues and organs (e.g.: the central nervous system (CNS), the brain and spinal canal, and bone marrow) (3);
- Plasticity – must be flexible enough to be able to remodel in response to external forces, but should also be rigid and resilient to forces (4);
- Maintenance of mineral homeostasis, serving as a reservoir for storing minerals – calcium and phosphate ions (3,5-7). The bone contains 99% of the body's calcium and acts as a reserve of this ion;
- Provides the proper environment for hematopoiesis within the bone marrow spaces (6,7).

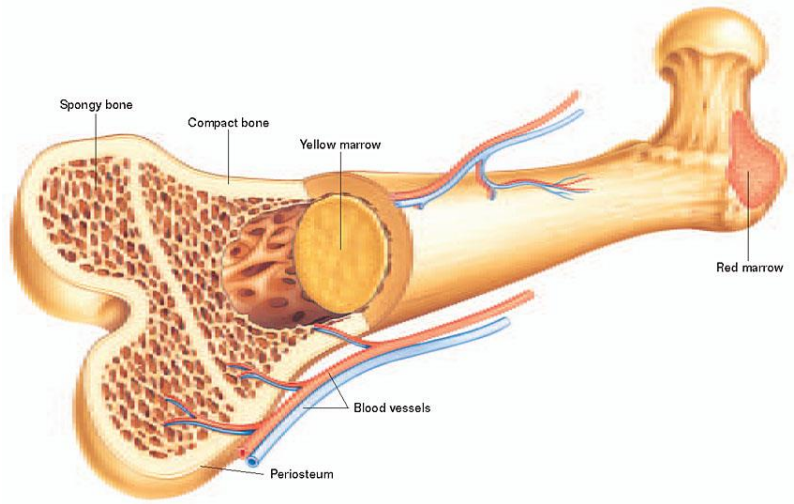


Figure 1 – Structure of bone tissue in a long bone **(8)**.

CLASSIFICATION

Macroscopic Classification

Bones can be classified regarding the form, being characterized in long, short and flattened bones (3,7). The first ones have greater length than width, and most of the bones of the upper and lower members are of this type; the second ones exhibit the length similar to the width; the last ones have a thin and flattened shape and are, as a rule, rounded (3,9).

The long bone comprises three components: the diaphysis that corresponds to the bone's body; the epiphysis are the edges of the bone; and the epiphyseal plate is composed of cartilage that ossified and becomes the epiphyseal line (3).

Microscopic Classification

At the microscopic level, the bone tissue can be classified as cortical or compact bone, with high density and rich in mineralized bone matrix, and trabecular or cancellous bone, constituted by small cavities surrounded by bone matrix (Figure 2) (3,10). These two types of bone are formed by a lamellar pattern, in which the collagen fibrils are placed in alternating directions (7).

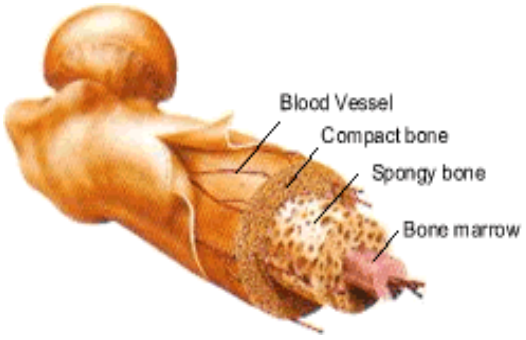


Figure 2 – Schematic representation of the bone tissue – cortical and trabecular bone **(11)**

The cortical bone is approximately 80% of the bone tissue existing in the body and is located in the diaphysis of long bones (1,10) and on the surface of the majority of bones (Figure 3). It is a dense bone due to the compaction of the collagen fibers (3). It's organized in a Haversian system (Figure 4) (7) that consists of a central canal, the Haversian canal, which is surrounded by concentric bone, *lamellae*. Inside of these *lamellae* are the osteocytes within small spaces, *lacunae* (3,7). Inside are the blood vessels, which are responsible for osteocytes, nerves and connective tissue nutrition (7).

The cortical bone has two surfaces, the endosteum and the periosteum (7). The periosteum is a membrane of connective tissue that covers the outer surface of the bone and has a high osteogenic potential (1,3). The endosteum has a similar structure and it's located in the inner surface of long bones. It consists of a single cell layer, including osteoblasts, osteoclasts and osteochondral progenitor cells (3). These two membranes play an important role in the nutrition of bone tissue and contain the major cells of bone metabolism. Thereby, have a significant contribution to bone remodeling and regeneration processes.

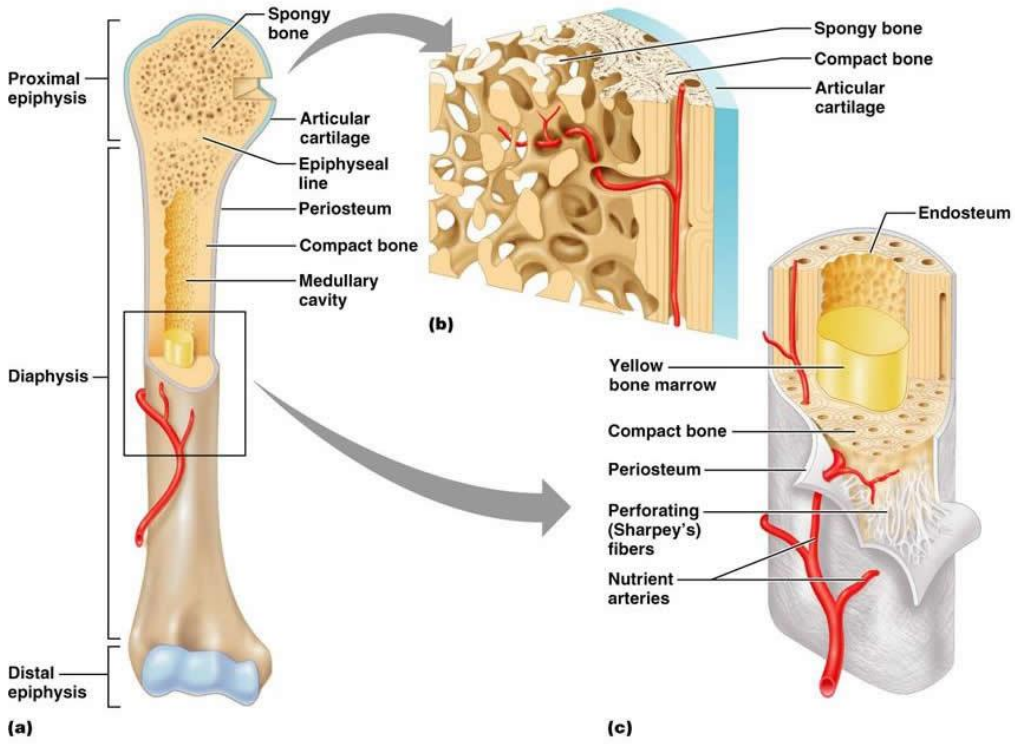


Figure 3 – Typical structure of a long bone. (a) Long bone components – epiphysis and diaphysis; (b) Epiphysis constitution – trabecular bone; (c) Diaphysis constitution – cortical bone **(12)**.

The trabecular bone is located inside the cortical bone, in the epiphyses of long bones (Figure 3) (10). It forms a network of bone plates connected to each other, the *trabeculae*, and the space between these is filled with bone marrow and blood vessels (Figure 4) (3). *Trabeculae* ensure the skeleton elasticity and stability. This type of bone is continuously remodeling, while cortical bone has a much slower renewal (13).

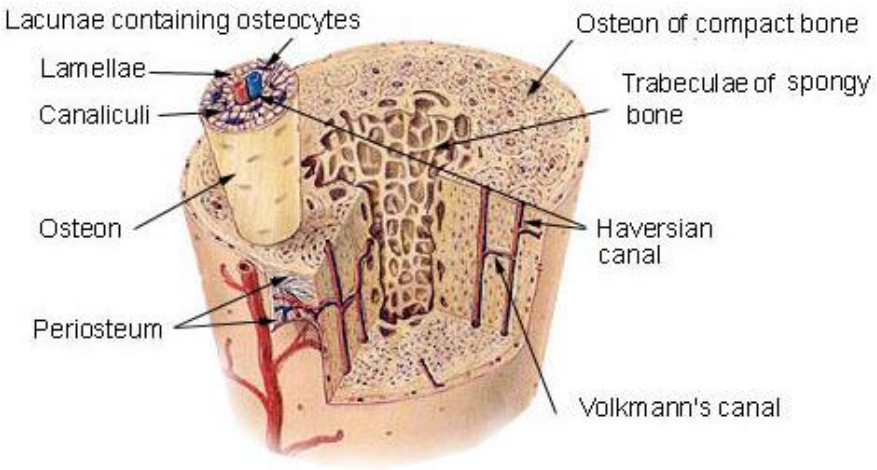


Figure 4 – Structure of cortical and trabecular bone tissue **(14)**.

BONE TISSUE FORMATION

The formation of new bone occurs during growth and in fracture repair (15,16). During development, there are two mechanisms responsible for bone tissue formation – endochondral ossification and intramembranous ossification (3).

Most of the bone tissue is formed by endochondral ossification (17), which takes place in the cartilage (3). Thus, it's formed a cartilaginous matrix that precedes the bone tissue formation. The cartilaginous cells differentiate into osteoblasts that will invade and secrete bony matrix (Figure 5) (17). On the other hand, intramembranous ossification is the bone formation *de novo* and occurs in connective tissue membranes (3,17). This is a formation way that derives directly from the mature osteoblast differentiation from mesenchymal condensations (Figure 6).

These two mechanisms cease when the adolescent growth is accomplished and mark a transition to adult bone remodeling once bone activity is concentrated on maintenance and repair.

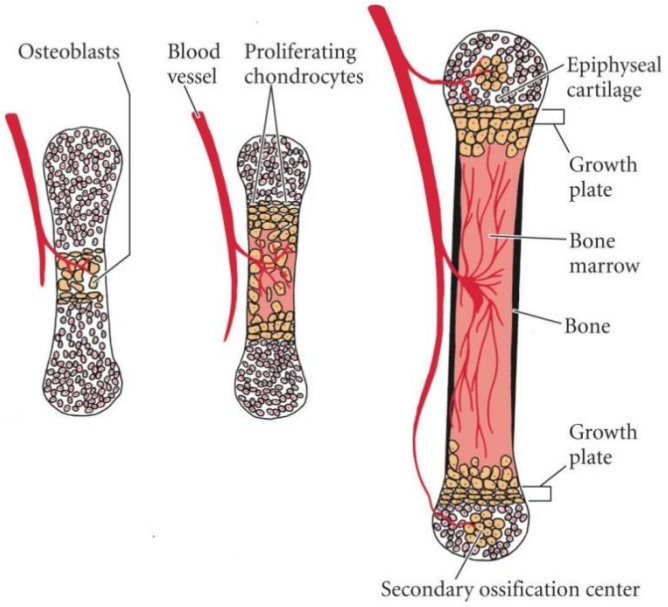


Figure 5 – Schematic representation of the endochondral ossification process **(18)**.

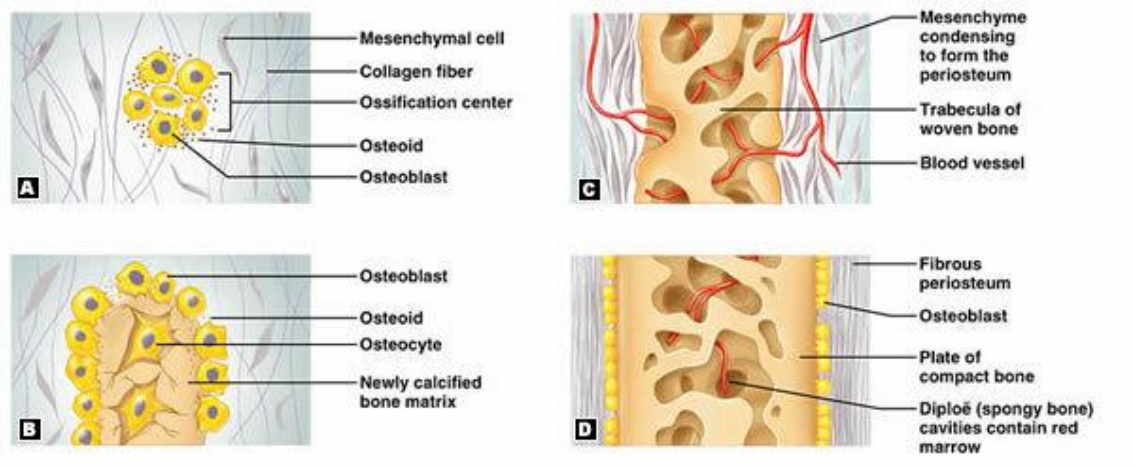


Figure 6 – Schematic representation of the intramembranous ossification process **(19)**.

In both processes, the initially formed tissue is characterized as primary or immature bone tissue, in which collagen fibers form clusters arranged in an irregularly shape and without a defined organization. This is a temporary tissue that will be gradually replaced by secondary or lamellar bone tissue, which has an ordered cell and matrix arrangement.

BONE CELLS

Bone tissue possesses various cell types that have origin in two distinct lines (Figure 7):

- Cells from osteoblastic lineage – osteoblasts, osteocytes and lining cells – derived from mesenchymal stem cells (MSC) (2,15,20,21);
- Osteoclasts derived from bone marrow hematopoietic cells (2,17).

The development and differentiation of osteoblasts and osteoclasts are controlled by growth factors and cytokines produced in the bone marrow microenvironment and by adhesion molecules which mediate cell-cell and cell-matrix interactions (2).

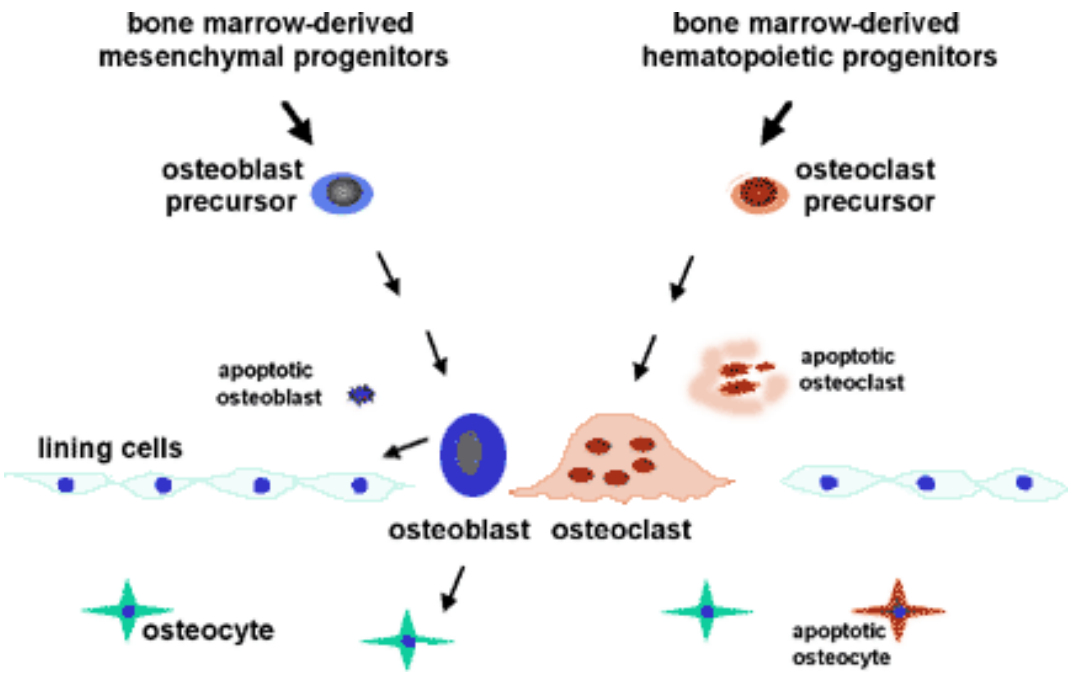


Figure 7 – Bone cell formation originated from two distinct lines: cells derived from mesenchymal cells – osteoblastic lineage cells (osteoblasts, osteocytes and lining cells), and cells derived from bone marrow hematopoietic cells – osteoclasts **(22)**.

Osteobastic Lineage Cells

The osteoblast lineage cells are responsible for the bone tissue formation during embryonic development, growth, remodeling, fracture repair or any bone defect. The differentiation process starts with an osteoprogenitor and then there's a progressive differentiation – pre-osteoblast, osteoblast and osteocyte (Figure 8).

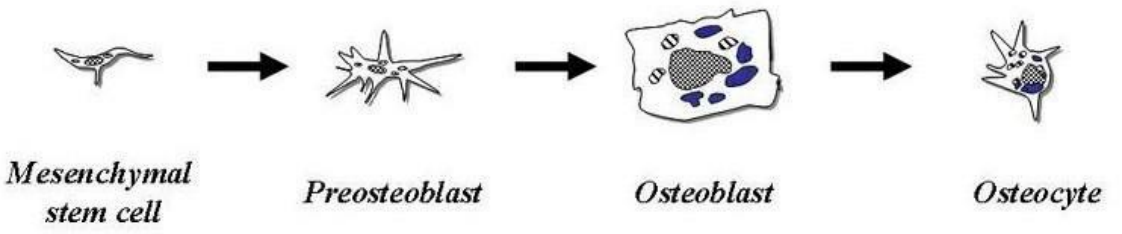


Figure 8 – Differentiation of osteoblasts and osteocytes. The pre-osteoblasts slowly differentiate from MSCs, in the bone marrow, to adopt an osteoblastic phenotype. As the osteoid will be mineralized by osteoblasts, these are deposited as osteocytes in *lacunae* **(23)**

Osteoblasts are the cells responsible for the organic matrix formation – osteoid – and subsequent mineralization, the process of hydroxyapatite (HA) deposition (2). The formation of HA crystals and subsequent bone matrix mineralization occurs when the content of these vesicles is released by exocytosis (3).

They have a cuboid shape, basophilic cytoplasm, are mononuclear cells and have a limited half-life period (about 3 months) that can result in three different targets: apoptosis; being engaged by the mineralized extracellular matrix (ECM) and become osteocytes, or differentiate into lining cells (24,25). Osteoblasts are arranged side by side on bone surfaces in a similar way to a simple epithelium and are capable of transdifferentiate into adipocytes, myoblasts, chondrocytes and osteoprogenitor cells under appropriate conditions (15,16).

There are numerous transcription factors responsible for the commitment of pluripotent mesenchymal cells into the osteoblast cell lineage. Core-binding factor α1 (Cbfa1), Runx-2 and Osterix (Osx) are the most important (26). Cbfa1 is a transcription factor of the *runt*-domain gene family and plays a critical role in osteoblast differentiation (27). Runx-2 is also a *runt*-domain gene and is involved in the production of bone matrix proteins (27). Osx is essential for osteoblast differentiation at an early stage but it also inhibits osteoblast differentiation at a later stage (27).

Osteoblasts play an important role in the bone formation process and in the control of bone remodeling. During bone formation, mature osteoblasts synthesize and secrete collagen I (Col I) and several non-collagen proteins such as osteocalcin (OC), osteopontin (OPN) and bone sialoprotein (27). Osteoblasts also synthesize insulin-like growth factor-I (IGF-I), interleukin-1 (IL-1) and IL-6. IGF-I secreted from osteoblasts in the bone tissue has been demonstrated to be a potent chemotactic factor that might play a main role in the recruitment of osteoblasts during bone formation (27). Apart from IGF-I, IGF-II, transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) are other examples of external regulators of osteoblast function (28).

These cells are responsible for the production of bone tissue specific proteins, cell adhesion proteins, bone growth factors, proteoglycans and for storing enzymes, calcium and phosphate ions in vesicles.

The osteocytes are the most abundant cell type in bone – there are 10 times more osteocytes than osteoblasts and its half-life is about twenty years. They are considered the most mature osteoblastic cells and result from the process whereby the osteoblasts are surrounded by mineralized bone matrix and stop producing it (3).

These cells are regularly spaced throughout the mineralized matrix and communicate with each other and with cells on the bone surface via multiple extensions of their plasma membrane that run along the *canaliculi* (2). Osteocytes are surrounded by an ECM forming spaces called *lacunae*, which are connected to each other through channels that are occupied by osteocytes extensions (2).

The location of osteocytes makes them good candidates for mechanosensory cells able to detect the need for bone augmentation of the skeleton and the need for microdamage repair (2).

There is a direct relationship between the amount of osteocytes and the bone formation process. By this mean, the higher bone formation, the greater the amount of trapped osteoblasts, so the number of formed osteocytes will increase.

The lining cells are inactive osteoblasts with dense nuclei and a more flattened shape, which lie on the bone tissue surface. These cells have less cytoplasmic organelles than osteoblasts and its biological function is not well defined: seems to be responsible for the removal of the osteoid thin layer that coats the surface bone, thus exposing the bone to bone resorption performed by osteoclasts, and take part in typical osteoblasts functions under appropriate activation (25).

Osteoclastic Lineage Cells

Osteoclasts are giant, mobile and multinucleated cells with abundant mitochondria, numerous lysosomes and free ribosomes (2). These cells have the same origin as monocytes and macrophages and a short half-life period of approximately 2 weeks (Figure 9).

Osteoclasts are responsible for the bone destruction or resorption process, which occurs through the release of acidic substances that form an acid microenvironment to dissolve the bone tissue. This acidic environment is created by the action of an ATP-driven proton pump located in the ruffled border membrane (2).

The receptor activator of nuclear factor κB ligand (RANKL) is an osteoclast differentiation factor that binds to the RANK receptor in osteoclasts. Osteoprotegerin (OPG) is a soluble tumour-necrosis factor receptor that inhibits osteoclast differentiation by interacting with RANKL (26,27).

Osteoclasts have high amounts of a phosphohydrolase enzyme, tartrate-resistant acid phosphatase (TRAP), a characteristic that is commonly used for the detection of osteoclasts in bone specimens (2). These cells also participate in long-term maintenance of blood calcium homeostasis and are important in bone development and growth through the release of growth factors.

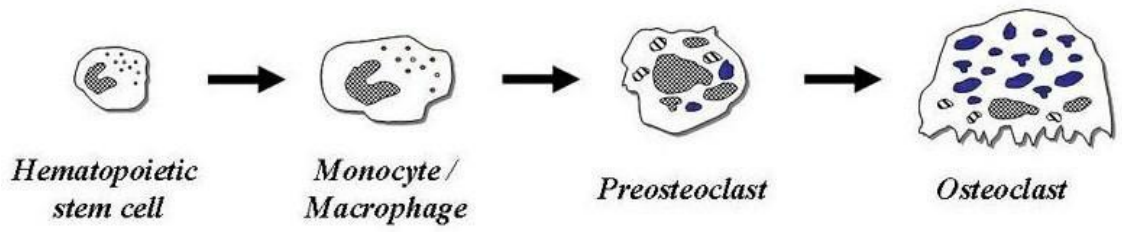


Figure 9 – Osteoclast differentiation. Osteoclasts are multinucleated giant cells that differentiate from monocyte/macrophage lineage hematopoietic cells in the bone marrow **(23)**.

BONE MATRIX

The bone matrix is composed of two different components, an inorganic component (50-70%) and an organic component (30-50%). The rest are lipids and water.

The organic material consists mainly of collagen which confers resistance to the matrix – types I, III and V, where the most abundant is type I – and proteoglycans (1-3,17). There are also non-structural proteins, such as growth factors, blood proteins, osteonectin and OC (7).

The inorganic material is responsible for the hardness of the bone matrix and is composed mostly by calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions that form crystals, also called HA crystals – $Ca_{10}(PO_4)_6(OH)_2$, when deposited in the matrix (1,3). These crystals are arranged along the collagen fibrils and are surrounded by proteoglycans and glycoproteins. Magnesium, sodium, potassium, bicarbonate and citrate ions also exist in the bone matrix but in lowest amount. The combination of HA with collagen fibrils gives the hardness and strength to the bone tissue that characterize it.

Calcium is the main mineral in the skeleton formation, acting in its structure, internal organs protection and locomotion. Moreover, muscle contraction, blood clotting and neuronal excitation mechanisms require calcium (7,29). Thereby, the calcium concentration must be kept within specific limits in the different physiological compartments.

The phosphate anion also exists in high concentration in bone tissue, being the major constituent of their crystalline structure. It is also an essential component of the membrane lipids and DNA, so it's crucial for cell signaling (7). The balance and homeostasis of plasmatic phosphate are the reflection of food diet, renal excretion and internal exchanges between the extracellular fluid, soft tissues content and skeletal reservoirs (30).

BONE REMODELING

The bone tissue is in constant remodeling, i.e. there is a continuous balance between resorption and formation of new bone (25,31,32), regulated by the action of osteoclasts and osteoblasts, respectively (1,2,5,15,33). Bone remodeling is a physiological process that maintains the skeleton integrity (34). Through this process, about 5% of cortical bone and 20% of trabecular bone is renewed per year (35). It can be said that it is the cellular basis of bone metabolism, thus a change in the process may lead into a metabolic disorder.

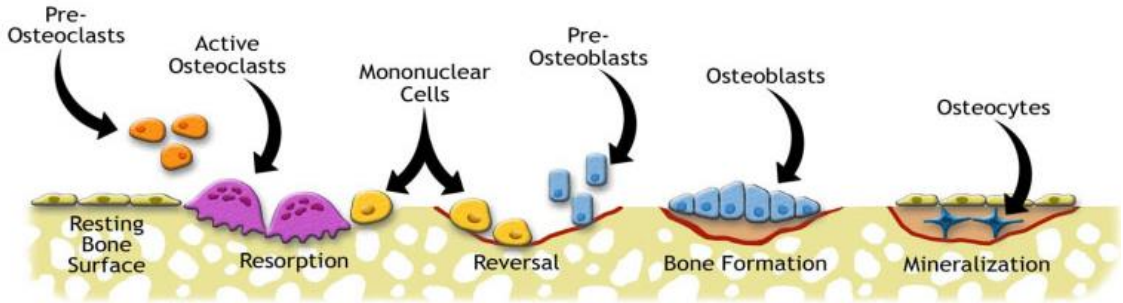


Figure 10 – Bone remodeling process **(36)**

At the microscopic level, the bone remodeling process takes place in small areas of the cortical and trabecular surface called basic multicellular units (BMUs), characterized by the coordinated action of osteoclasts and osteoblasts (2,27,35). BMUs are a unique temporary structure with about 6-9 months of lifespan. During this time numerous generations of osteoclasts (lifespan of about 2 weeks) and osteoblasts (lifespan of about 3 months) are formed. The BMU comprises a group of osteoclasts in the front, a group of osteoblasts in the rear, a central vascular capillary, a nerve supply and associated connective tissue (2).

A bone remodeling cycle consists of four different and sequential phases – activation, resorption, reversal and formation.

Bone remodeling begins with the activation of resting cells to the resorption place, including osteoclastic precursors and bone-marrow mononuclear monocyte-macrophages, which differentiate into multinucleated cells and active resorbing osteoclasts that begin the resorption process (27). In these phase the osteoclasts begin to dissolve the mineral matrix and decompose the osteoid matrix. There is also the release of growth factors contained within the matrix, essentially TGF-β, PDGF, IGF-I and IGF-II (35). After the bone resorption, osteoclasts migrate from the resorption site that is then invaded by a heterogeneous population of mononuclear cells with macrophage and pre-osteoblastic cells.

When resorption is completed, it starts the reversal phase where the osteoclasts die through apoptosis and osteoblast precursors locally proliferate, differentiate into mature osteoblasts and migrate into the resorption lacuna made by osteoclasts (27).

The following stage is the formation phase, in which osteoblasts synthesize new osteoid material that fills the resorption lacuna and becomes mineralized in the resting phase (27,35). As the organic matrix is mineralized, it surrounds the osteoblasts, which lose their synthetic activity and are therefore called osteocytes.

The activation and regulation of bone resorption requires an interaction between osteoblasts and osteoclasts. The molecular mechanism that supports this relationship is explained by the RANK/RANKL/OPG system (Figure 11). RANK is a protein member of the tumor necrosis factor (TNF)-receptor superfamily and is expressed by mature osteoclasts, dendritic cells and some cancer cells (27). RANKL is a TNF superfamily member expressed by osteoblasts and is essential for the recruitment, differentiation, activation and survival of osteoclastic cells through binding to its specific receptor RANK (27). OPG is a soluble receptor of RANK and is synthesized by osteoblasts, stromal cells, vascular smooth muscle cells, B lymphocytes and articular chondrocytes (27).

By modulating RANKL and OPG, osteoblasts can control osteoclast differentiation and activity and consequently bone remodeling: RANKL binds to RANK that is present on the surface of osteoclast precursors leading to the activation of Nuclear Factor κB and transcription of genes involved in osteoclastogenesis. OPG has the ability to act as a decoy by binding to RANKL and blocking the RANKL/RANK interaction (2,27). This way, OPG inhibits osteoclastogenesis, osteoclast activity and bone resorption.

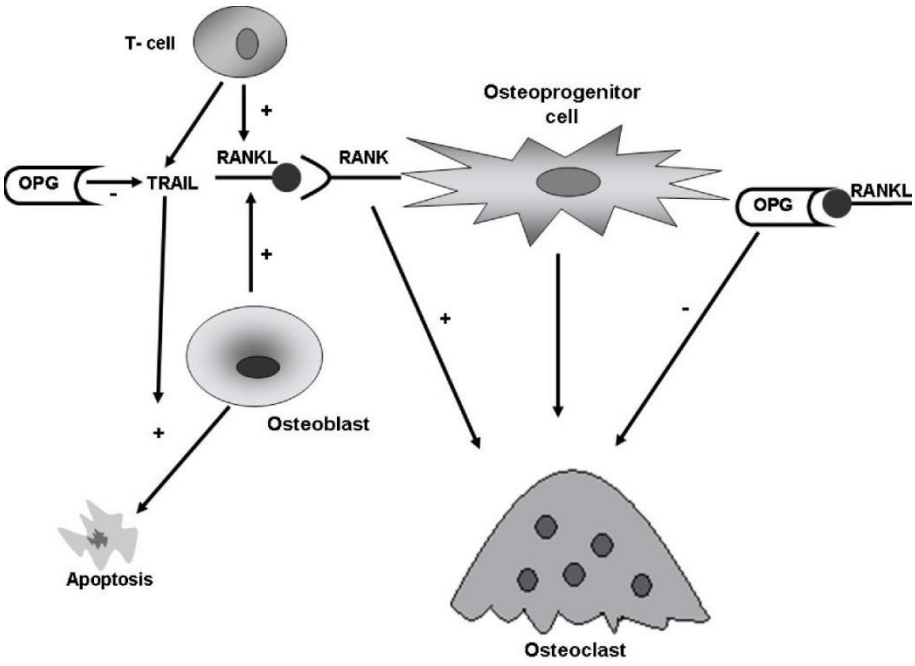


Figure 11 – Molecular mechanism of the RANK/RANKL/OPG system.

Bone remodeling is a key process in the normal body behavior because it gives the characteristics that bone needs to maintain physiological functions (15). Furthermore, bone remodeling replaces old bone with inferior biomechanical properties and helps maintaining the normal calcium and phosphate concentrations (37).

In young adults, the rate of bone resorption and formation are ideally equal, and the total bone mass remains constant, which do not occur in children, where bone formation rate is higher than bone resorption, since the bones are growing (7).

Bone remodeling increases in premenopausal and postmenopausal women being then attenuated with age in both genders, continuing to be faster than in pre-menopausal periods (7,38).

In pathological situations, it is common to see a change in the normal functioning of bone remodeling, i.e., an imbalance between the processes of bone formation and bone resorption, which leads to bone loss (39). Thus, there is an increase in the osteoclasts number and activity, the cells responsible for the bone resorption process, and/or a decreased differentiation of osteoblasts, which are responsible for bone formation (39). Osteoporosis is an example of a bone disease where this occurs.

BONE METABOLISM

Bone is a mineral that releases calcium and phosphate in response to hormones secreted by several organs. These elements are very significant in the bone composition, and the levels monitoring are essential to the performance of many vital physiological functions. Therefore, normal levels of calcium, phosphate and related minerals are maintained by a complex regulatory system. The major hormones that regulate these values are the parathyroid hormone (PTH) and vitamin D (38). The calcitonin, growth hormone (GH) and estrogens also play an important role in the regulation of bone metabolism.

The parathyroid hormone (PTH) is released by the parathyroid glands and its secretion is regulated by plasmatic concentration of calcium (13,29,40). The primary effect of PTH is to increase plasmatic calcium levels and reduce plasma phosphate concentration, acting in three main organs – the bone and the kidney, directly, and the gastrointestinal tract, indirectly (13,29,41). In the bone, PTH receptors are present in osteoblasts as well as in osteoclasts, and its effect in this tissue involves two mechanisms:

- An immediate stimulation consists in the activation of the bone cells present in the bone tissue. PTH binds to specific receptors in osteoblasts and osteocytes and activates a calcium pump, where calcium is transferred from the bone fluid to the extracellular medium. Calcium levels in the bone fluid are rapidly replenished by removing calcium phosphate salts from amorphous deposits existent along the osteoblastic cells;
- A slower effect, that can take several days or weeks, consists in the activation of the osteoclast system, with an increase in the osteoclasts recruitment and activity. This process will lead to an increased resorption of mineralized bone tissue.

Vitamin D in its active form – 1.25-dihydroxyvitamin D3 (calcitriol) – is a steroid hormone (35) and one of the major regulators of calcium metabolism. Its main functions are to stimulate intestinal absorption of ingested calcium and to promote the calcium resorption by the bones (13,40). In the bone, only osteoblasts have calcitriol receptors, despite vitamin D stimulate bone resorption. As a result, recruitment, differentiation, and fusion of precursors into active osteoclast increase. Vitamin D is also involved in the process of new bone formation by inducing the synthesis of osteocalcin in osteoblasts. Moreover, it has an action of negative feedback suppressing, in a direct way, the gene responsible for the PTH synthesis (13,40).

Calcitonin is produced in the parafollicular cells of the thyroid and its greater effect on bone metabolism is the reduction of plasma calcium concentration caused by the inhibition of bone resorption (28,35,41). By this way, it reduces the number and activity of osteoclasts (35). Hence, calcitonin is a physiological antagonist of PTH hormone in relation to calcium levels. However, their effect on phosphate concentration is similar, i.e. reduces plasma phosphate levels. The greatest stimulus for calcitonin secretion is the increase in plasma calcium (13,30), but vitamin D may also promote calcitonin levels by food intake, without increasing calcium concentration.

Growth hormone (GH) is produced and stored in the acidophilic cells of the anterior lobe of pituitary and promotes bone growth. GH increases both bone resorption and bone formation processes (13,30). Its action on cell growth requires the formation of peptides known as somatomedins, also called insulin growth factors (IGFs). These mediate the responses of GH on cartilage, bone tissue, muscle, fat tissue and fibroblasts (13). To highlight the effect of IGF-I on bone metabolism as it increases chondrocytes and osteogenic cells proliferation and proteins deposition by these two cell types. It also stimulates chondrocytes conversion into osteogenic cells leading to bone tissue formation (13).

Estrogens are hormones synthesized by the ovaries and have an important role in maintaining bone mass in women. They promote bone tissue formation (13). When estrogen levels are reduced there is an increase in the intensity of bone remodeling (4), which causes an imbalance between osteoblastic and osteoclastic activities. So there is an increase in osteoclasts activity and a decrease in osteoblasts activity, which leads to the formation of resorption spaces that are not filled by immature bone. Estrogens bind to specific receptors in the cytoplasm of bone cells, which results in formation and release of soluble factors with autocrine and paracrine actions in both osteoblasts and osteoclasts. This will influence their recruitment, proliferation, differentiation and metabolic activity (4).

BONE REGENERATION

Bone is a highly specialized and dynamic tissue that has the capacity of self-regeneration. During development and growth bone undergoes a modeling process in which it is removed from one site to a different one (2). The bone regeneration is a process that takes place throughout life to replace the old bone with new and it has special importance when a fracture occurs. This is more common in bone pathologies, or by trauma, osteonecrosis and tumors. A fracture is a break in a bone that is always accompanied with damage in the adjacent tissues (42). In cases of bone tissue loss it is necessary the immediate stimulation of new bone production.

There are a number of events that follow a bone fracture. They usually start with the formation of a wound, followed by the healing and finally the repair process.

The injury process begins with the stimulation of growth and differentiation factors which then activate localized pluripotent osteoprogenitor cells. These cells produce bone morphogenetic proteins (BMPs) that induce the migration of mesenchymal cells, which in turn proliferate and differentiate into bone-forming cells (42).

Afterward, it starts a rapid inflammatory response resulting in tissue edema and chemotaxis through the release of cytokines and growth factors. Then follows the first phase of collagen repair that involves the deposition and formation of granulation tissue, which becomes a new and temporary weak tissue. This process ends with the second stage of collagen repair that results in extracellular matrix remodeling, localized angiogenesis and reproduction of full-strength tissue. Bone remodeling is initiated with both endochondral and intramembranous ossification which are stimulated by hypoxia and vascular disruption at the fracture site (17,42).

The above coordinated processes result in the reconstruction of normal bone and restoration of a structural unit. However, these normal bone regenerative mechanisms breakdown during failure of optimal bone remodeling and repair, or in large bone fractures and defects.

In these cases, it is necessary to resort to Tissue Engineering: an interdisciplinary field that applies the principles of engineering and the life sciences to the development of biological substitutes that restore, maintain, or improve tissue function (43). This is a new field of research that suggests the regeneration of the tissue instead of its substitution (44) and it has gained special attention due to the lack of clinical treatments capable of restoring full functionality. For that the use of grafts for bone repair is a good option.

The autologous graft (graft from the individuals themselves) is the clinical gold standard, but although effective, with no immunogenicity, superior grafting and minimal risk of disease transmission, it has a limited supply of viable donor tissue, there is the need of additional surgeries, increased risk of infection and donor site morbidity (45,46). Allograft (graft from another individual) bone is an alternative to autografts but it also has some disadvantages related to limited donor supply, disease transmission and inadequate physiologic and biomechanical responses (46).

The biologic mechanisms that provide a basis for bone grafting are osteoconduction, osteoinduction and osteogenesis. This way, the bone graft material must be osteoconductive serving as a structure (scaffold) that allows cells to migrate and function within its limits (46); osteoinductive providing factors that stimulate the proliferation and differentiation of osteoprogenitor cells into osteoblasts that then begin the new bone formation (46); and osseointegrative being capable of integrate into the surrounding tissue (46).

Thus, the superposition of two or more materials in order to completely achieve these characteristics is a logical strategy. In effect, the creation of composites is a biomimetic approach, as bone can be viewed as a composite of collagen, the principal organic component; HA, the inorganic mineral component; water; and small amounts of other organic phases.

OSTEOPOROSIS AND OSTEOPENIA

Osteoporosis is a pathological condition characterized by reduced bone mineral density (BMD) and microarchitectural changes in bone tissue that result in bone fragility and increased risk of fracture (2,31,32,47). A pathological imbalance between bone resorption and bone formation during the remodeling process may be the cause of this disease (31,39,48,49). Its most obvious morphological characteristic is bone loss, which tends to be more evident in skeleton areas with a high content in trabecular bone (Figure 12).

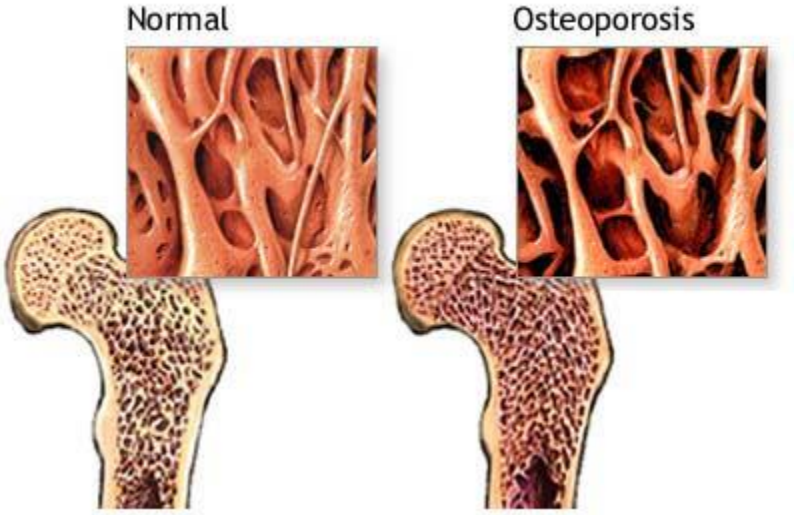


Figure 12 – Morphological difference between a bone in physiological conditions and an osteoporotic bone **(50)**.

There are two classic forms of osteoporosis, the primary or physiological osteoporosis and the secondary osteoporosis (32), that is caused by other associated conditions. The first one can be divided into two forms:

- Postmenopausal osteoporosis – often identified in women aged over 50 years (47,51) and characterized by high bone resorption rate resulting in an accelerated and increased osteoclastic activity caused by estrogen deficiency (52);
- Senile osteoporosis – affects individuals of both genders with ages over 70 and has a normal or slightly increased bone resorption, associated with a decrease in the osteoblast activity that leads to low bone formation (52,53).

As stated earlier, secondary osteoporosis can be caused by several diseases that lead to skeleton weakness like genetic disorders – *osteogenesis imperfecta* and osteoporosis-pseudoglioma syndrom; endocrine disorders – Cushing's syndrome, hyperparathyroidism, hypothyroidism and hypogonadism, and inflammatory or nutritional disorders – rheumatoid arthritis, Crohn's disease and celiac disease (38,52).

Several systemic and local factors, both in physiologic and in pathological conditions, can influence the rigorously coupled activity of osteoblasts and osteoclasts, determining an imbalance in bone remodeling in favor of resorptive activity (27). As previously described, the RANK/RANKL/OPG system represents the main regulatory factors of the remodeling process. Some studies have demonstrated that this system is involved in the pathogenesis of osteoporosis (54,55).

The peak bone mass is reached at the age of young adults, but during the third or fourth decade in both genders, bone resorption begins to exceed bone deposition. This bone loss associated with age is a common biological phenomenon and generally occurs in areas with high content of trabecular bone, such as the spine and neck of the femur. Thus, these sites are more prone to fractures in patients with osteoporosis. However, the rate of bone loss in each remodeling cycle can be anticipated by postmenopausal status, with women being more vulnerable to this condition and consequent complications.

Osteopenia is a condition where BMD is lower than normal and is broadly considered by clinicians to be a precursor to osteoporosis. Nonetheless, not every individual diagnosed with osteopenia will embrace the development to the pathological state of osteoporosis. As a convention, the osteopenic condition is defined when the bone density is set one standard deviation below that of an average 30-year-old white woman; while osteoporosis is defined when this value reaches 2.5 standard deviations or more below the reference standard (56). Despite the controversy regarding the definition and the clinical usefulness of osteopenia, since it is an abstract concept, without any bio-pathological basis, it is highly regarded by clinicians as a precursor state of osteoporosis. In fact, anti-resorptive treatment is broadly initiated in this state, aiming to delay the onset of osteoporosis (56).

EPIDEMIOLOGY

Osteoporosis is a disease with serious interest and concern since there is a high predisposition to fractures that cause pain, disability and, in some cases, premature death (37,57). There are several types of osteoporotic fractures, the most common occur in the vertebrae, wrist, femoral neck, distal radius and hip, and its epidemiology varies with age, sex and geographic location (37,58).

The epidemiology of osteoporotic fractures is based on some general principles: the majority of fractures is related to low bone mass levels; the risk of a fracture is higher in women than in men; the risk of a fracture increases with age; it's more likely to experience a fracture in patients who have suffered fractures in the past; vertebral fractures are very common, causing pain and disability, and are difficult to identify; vertebral fractures, with or without symptoms, indicate high risk of future fractures; the hip fracture is considered the most important type of fracture; the fracture risk may be genetically determined; several risk factors are related to decrease of bone mass (37).

Osteoporosis affects more than 200 million people worldwide (59) and reveals a high rate of both mortality and morbidity in Western countries like Asia, Latin America, Middle East and Africa. This is a disease that affects men and women, although the risk of an osteoporotic fracture occurs in women is 2-4 times higher than in men (48). It is estimated that about 13% of men and 40% of women aged over 50 will suffer an osteoporotic fracture during her life time. These values increase to 22% in men and 47% in women taking into account future mortality trends.

PATHOPHYSIOLOGY

Under physiological conditions, in an adult there is a balance between bone formation by osteoblasts and bone resorption by osteoclasts, in a dynamic process of bone remodeling. Osteoporosis occurs when there is a disturbance in this process and the balance tends in favor of bone resorption. This is a multifactorial disease and any model for its pathogenesis has to recognize a number of different mechanisms (32), shown below.

- Age-related changes – during aging, the rate of bone remodeling increases in both genders (32). The amount of bone resorbed is higher than bone formed (4), and in the tissue level, osteoblasts formation is impaired compared to osteoclasts action, due to the decrease in number and activity of osteoblastic cells. These osteoblasts related disorders may be caused by extrinsic mechanisms which are mediated by changes in bone microenvironment, such as changes in level of hormones and growth factors, and intrinsic mechanisms caused by cellular senescence of osteoblasts. As a result, the formation of cortical and trabecular bone undergoes a decrease with age. The several growth factors deposited in the ECM also tend to become less potent with time. However, while the synthesis of new bone decreases with aging, the activity of osteoclast-mediated resorption remains unchanged.

- Hormonal influences – the reduction of estrogen levels is associated with menopause (13,32,49,51), with an annual fall of about 2% of cortical bone and 9% of trabecular bone. Thus, it is not surprising that about half of postmenopausal women suffer osteoporotic fractures, when compared with the number of men in the same age group.

  Bone loss is more common in early postmenopausal women since the suppression of estrogen leads to an increased bone remodeling rate (4), creating many focus of bone remodeling in the endosteum. This deficit in estrogen promotes osteoclast activity because probably suppresses its apoptosis (52). The effect of decreasing estrogen level may be in part attributed to increased production of cytokines – particularly IL-1 and TNF (52). In addition, estrogens regulate resorption by limiting the release of RANKL by osteoblasts. The loss of estrogens leads to a significant increase in the expression of RANKL, which in turn results in excessive formation and activity of osteoclasts. At the same time, the levels of OPG increase with age, and it is possible that this production increases as a homeostatic response to limit bone loss which occurs with an increase

of other factors of bone resorption. However, despite some osteoblastic activity occurs simultaneously, the anabolic response seems to be insufficient to maintain the pace since bone resorption by osteoclasts is so high.

The increase of osteoclasts activity promotes bone erosion by the cells helping to trabecular penetration and rupture of bone architecture. At each remodeling site, the amount of bone resorbed is greater than the replaced bone, producing a negative balance in the BMU, which is the basis of bone loss. Moreover, the lack of estrogen increases the prevalence of apoptosis, which may impair the ability of osteocyte to repair damage and thus contribute to promoting bone fragility. The fast initial drop in BMD is the result of the increase in porosity of many BMUs and occurs due to the normal delay in the onset of bone formation and due to the slow filling within various resorption cavities. The deterioration of microarchitecture may reflect the intensity and duration of osteoclastic activity and the number of osteoclasts formed.

The increased remodeling and the negative bone balance lead to bone loss, trabecular narrowing and connectivity loss, cortical erosion and porosity. Later, a greater quantity of mineralized interstitial bone, away from the surface remodeling, accumulates damage on a microscopic scale, while the most superficial bone is replaced by younger and less mineralized bone, reducing the hardness. The bone remodeling by periosteal apposition reduces compressive pressure by distributing loads over a larger area and retains, in part, the torsional force. Additionally, it can be impaired by abnormalities in the function of periosteal osteoblasts or osteocytes signaling.

- Lifestyle – studies have shown that alcohol intake is a risk factor for bone loss, but it was concluded that the weight, the use of estrogen and tobacco are also important predictors for bone health (38). It is not known whether the reduction in BMD found in alcoholics is mostly due to an inadequate food intake, a lack of physical exercise or a direct effect of alcohol (32,60) in the reduction of osteoblastic activity. However, the most common is being a combination of all of these factors.

  The tobacco is also associated with several risk factors for osteoporotic fracture, such as a reduction in bone mass peak, early menopause and thinness. Apparently, smoking reduces BMD by a mechanism independent of its effect on weight or estrogen metabolism and can act to reduce calcium absorption.

  Physical activity is also important because it is known that the mechanical forces stimulate bone remodeling. Consequently, a decrease in physical exercise increases bone loss. This reduction in physical activity in older individuals may also contribute to senile osteoporosis. As the magnitude of skeletal loading has greater influence on bone density than the number of charge cycles, the type of physical activity is important. Thus, resistance exercises such as weight training leads to a more effective increase in bone mass than endurance activities like jogging.

- Genetic factors – several population and genetic variations play an important role in the peak bone mass and changes in BMD. For example, the peak bone mass is higher in men than in women and in African people when compared with Caucasian or Asian individuals (38,61). In fact, genetic changes allow us to explain the high percentage of BMD variation. Polymorphism in vitamin D receptor may be responsible for approximately 75% of the variation in the peak bone mass achieved in a given individual (61). Other genetic variables can influence the cellular uptake of calcium or PTH synthesis and thus affect biological related responses.

- Calcium nutritional state – the calcium absorption by food intake may determine the status of bone metabolism (38). In the world population is of particular relevance that most teenage girls have inadequate dietary intake of calcium in the period of fast bone growth. As a result, they do not achieve their peak bone mass that would be expected, being more susceptible to develop clinically significant osteoporosis at an early age (62).

  1.25-Dihydroxyvitamin D, the active form of vitamin D, have important physiological activities like calcium absorption in gastrointestinal tract, regulation of calcium manipulation in the kidney, regulation of homeostatic control of bone remodeling, and synthesis regulation of the parathyroid hormone. If there is an abnormality in vitamin D formation, metabolism or action may occur an increase of bone resorption and therefore a more rapid bone loss (38). Subclinical deficiencies of vitamin D have been found in osteoporotic women. Vitamin D can also directly influence muscle strength and dexterity.

- Glucocorticoids – chronic glucocorticoid therapy is known as one of the major causes of osteoporosis. High doses of glucocorticoids and long exposure periods to corticosteroids inhibit osteoblast proliferation and activity and enhance osteoblast and osteocyte apoptosis. They also enhance bone resorption by increasing the RANKL expression and reducing the production of OPG (27).

Overall, the mechanisms described above can be grouped into three categories:

- Failure to achieve optimal peak bone mass and strength – largely determined by genetic background, but can also be affected by lifestyle;
- Faster bone loss due to resorption;
- Weakened response to bone formation during the remodeling process – which physiologically occurs shortly after achievement of peak bone mass, but can be greatly enhanced by pathological mechanisms.

Osteoporosis can also develop in connection with several pathological conditions – secondary osteoporosis – as stated above. The causes which lead to this type of osteoporosis include effects caused by prolonged therapy with glucocorticoids that increase bone resorption and decrease their synthesis (63).

DIAGNOSIS

Osteoporosis is difficult to diagnose because it remains asymptomatic until the occurrence of a fracture (38). In the past, it was often diagnosed in postmenopausal women and older men, based on radiological findings and in the presence of small fractures. Recently, it was established an inverse relationship between BMD and fracture risk, which allowed making the diagnosis based on BMD measurements that enable disease identification even before a fracture occurs (62,64).

Radiography

In old times, radiographs were used to evaluate the peripheral skeleton BMD, usually at metacarpals level. The metacarpal cortical thickness was thus used for many years for diagnosing and predicting the risk for osteoporosis. However, the sensitivity of this radiographic technique is poor (65), and metacarpal measurement results do not reflect the BMD at most important sites such as the hip and spine (61).

Radiographic analysis reveals bone loss recognizable only when 25-30% of bone density is lost, when osteoporosis is considered being in a more developed state. Although there is a correlation between BMD in central and peripheral skeleton, the association is not strong enough to predict central BMD measurements from a given peripheral bone (61). Nowadays, the main role of radiography is the diagnosis of secondary fractures to osteoporosis.

BMD Measurement

Quantitative analysis of bone density can be performed by several methods, among which we highlight the Dual-Energy X-Ray Absorptiometry (DXA), Quantitative Computed Tomography (qCT) and Quantitative Ultrasound (QUS).

Dual-Energy X-Ray Absorptiometry (DXA)

Dual-energy X-ray absorptiometry (DXA) is a very precise and accurate technique for assessing bone density at several skeletal sites (66), that determine the absorption of two beams of photons at two different energies (67). With this method it is possible to measure BMD (mass/area) in proximal femur and lumbar spine, as well as the mineral density throughout the body. However, it fails to differentiate between measurements of cortical and trabecular bone.

This method allows the BMD measurement in the hip or in the spine, with greater accuracy than other methods (accuracy error: 0.5-2%). The scanning time is about 5 minutes at each spot and the radiation dose is low (68,69). Besides the evaluation of normal anatomical sites, it may be used to provide side views of vertebral morphometric evaluations to determine vertebral fractures and deformities.

The results are reported as a density measurement in g/cm^2 and T and Z scores. The T scores represent the number of standard deviations below the mean BMD value for young adults (20-30 years old) (66). This is used to make diagnosis of normal bone density and with osteoporosis, in postmenopausal women and in men from the age of 50. The Z scores represent the number of standard deviations above or below the mean BMD value for subjects of the same age (66). These values are rather used to assess bone loss in premenopausal women and men younger than 50 years old.

The World Health Organization (WHO) (70) defines osteoporosis as a result of BMD evaluation of standard deviation of 2.5 or higher. The WHO continues to seek a new algorithm for defining the limits of osteoporosis treatment that includes other factors such as age. Osteopenia is an intermediate category of bone loss defined as a T-score between 1 and 2.5 (71). It’s a physiological condition characterized by the decrease of BMD.

Quantitative Computed Tomography (qCT)

Quantitative Computed Tomography (qCT), with appropriate software, allows determining the absorption of different calcified tissues so that areas of interest as the vertebral body can be studied. This technique has the capability of evaluating bone density in three dimensions (g/cm^3) (66). qCT has the advantage of distinguish trabecular from cortical bone. In addition, it can provide details related to the bone structure, including width and density in specific sub regions of cortical bone (66). The high resolution CT enables the measurement of trabecular diameter, inter-trabecular spaces and can identify abnormal trabecular architecture.

Recent developments of tridimensional CT (3D) allowed the evaluation of the 3D trabecular structure and could improve the ability to estimate biomechanical properties of the bone. Dual energy scanning (with twice the dose of radiation) can improve accuracy but the precision worsens. The radiation dose and the price are considerably higher than those of conventional qCT.

Quantitative Ultrasound (QUS)

Quantitative ultrasound (QUS) provides information regarding bone mass and structural organization of the bone. It assesses the speed of sound or the absorption pattern of different sound wavelengths designated attenuation of ultrasound bandwidth (66). The attenuation of ultrasonic signals during the passage through the bone may be measured by determining the reduction in signal amplitude of ultrasounds.

The ultrasound instruments have theoretical advantages in relation to DXA because they do not need radiation, are portable and cheaper. However, currently, it is difficult to apply the ultrasound to the clinic due to the lack of specific diagnosis criteria and the need to use manifold instruments. Moreover, as there are technical differences between devices, the results cannot be extrapolated from one device to another (66).

PREVENTION AND TREATMENT

Osteoporosis is a bone disease that affects a large number of people at ever earlier ages. Thereby, the main goal is to identify individuals at risk of developing osteoporosis and provide them a safe, effective and economical intervention to prevent appearance of this disease. However, the factors that contribute to the occurrence of osteoporosis are very heterogeneous and there are a large number of genetic parameters that are unknown, making it difficult to create a complete plan for its prevention and treatment (52).

Peak bone mass and resulting BMD are important throughout the growth and aging, which means that must be paid special attention to procedures that can optimize the level of bone mass (52). Thus, the approaches used for treatment and prevention of osteoporosis focus on prevention of bone loss. This can be done by several mechanisms:

- Increase of calcium levels to reduce bone loss – calcium and vitamin D supplements (29,37);
- Decrease in osteoclasts action to suppress bone resorption through pharmacological treatments – calcium, biophosphates, estrogens or analogs and calcitonin (29,37);
- Increase in osteoblasts action to promote bone formation – low doses of PTH and testosterone (29,37).

Prevention of this disease is also related to lifestyle, so it is encouraged the practice of regular physical exercise that is beneficial to maintain bone mass and reduce fracture risk (29,37,52); the reduction in alcohol, salt, caffeine and tobacco consumption, and nutritional care, such as increased consumption of animal protein and adequate calcium intake that maintains bone mass (37,52).

With use of continuous treatments, probably indefinitely, it is possible to decrease the risk of fracture or at least keep it down. This strategy can be changed with the emergence of agents that substantially increase bone mass and restore microscopic architecture of the bone (37). However, the treatment of osteoporosis is difficult, both theoretical and practical, and the efforts that imposed directly on prevention measures are continuous.

TETRACYCLINES

Tetracyclines are broad-spectrum antibiotics which act at the ribosomal level and interfere with protein synthesis (72). They were first used in the treatment of acne in the early 1950s and more recently have been considered for the biologic management of distinct conditions ranging from inflammation, proteolysis, angiogenesis, apoptosis, metal chelation, ionophoresis and bone metabolism. At the present, are available three groups of tetracyclines – natural-derived products, semisynthetic compounds, and chemically modified tetracyclines (CMTs) (72). Tetracyclines can act in several clinical conditions that include periodontitis, osteoporosis/osteopenia, rheumatoid arthritis, cancer invasion and metastasis, corneal ulceration, abdominal aortic aneurysms, inflammatory skin diseases and other immune-inflammatory conditions (73).

Upon administration, tetracyclines are widely distributed into the tissues and can also achieve the cerebrospinal fluid. Besides that, as they chelate with the calcium ions, they are concentrated at mineralized tissues, namely bone and teeth.

Tetracyclines and their derivatives are responsible for several actions that are independent from their antibiotic activity. Many of the non-antimicrobial properties may be related with their capacity for divalent cation quelation (74). These drugs proved to be effective against a range of mediators of the inflammatory cascade. Several direct or indirect mechanisms have been proposed – suppression of neutrophilic migration and chemotaxis, inhibition of T cell activation and consequent inhibition of their proliferation, inhibition and increased degradation of nitric oxide syntheses and pro-inflammatory cytokines inhibition. Tetracyclines can modify the expression of these mediators which makes them attractive for therapeutic action.

The anti-inflammatory actions include the inhibition of matrix metalloproteinases (MMPs) (75). MMPs are extracellular enzymes that rely on the availability of two cations per molecule to fulfill their enzymatic activity (76). Some MMPs (MMP-1, MMP-8 and MMP-13) are known as collagenases due to their capacity to break down fibrillar collagens. Others (MMP-2 and MMP-9) are known as gelatinases and are responsible for the degradation of collagen type IV, which can be found in the basement membrane. The MMPs family plays a role in angiogenesis, the process of formation of new blood vessels from pre-existing ones. Recent studies have shown that collagenases and other MMPs may participate in the degradation of type I collagen, the main component of the bone organic matrix and in the destruction of other connective tissue constituents. Thus, preliminary *in vivo* research has shown that tetracyclines were able to inhibit bone loss through inhibition of osteoclast-mediated bone resorption, but also by enhancing osteoblast activity, up regulation of type I collagen expression and increased bone formation (77,78).

Other important non-antimicrobial property of tetracyclines and related molecules is associated with their eventual antiapoptotic effect. Furthermore, the interaction between tetracyclines and the bone system is known for a long time. These agents have been used as diagnostic markers for a long time (79).

Overall, tetracyclines have been shown to inhibit MMPs, retard proliferation, induce apoptosis, and impair mitochondrial function in various experimental settings (80). Since the mid-twentieth century, they have found application beyond their anti-microbial activity in both the clinic and biomedical research; therefore, several translational studies were performed to demonstrate the clinical validity of it. After some research approaches, it was developed a low dose therapeutic regimen of doxycycline that showed to be non-antimicrobial, effective and safe. In this therapeutic regimen, doxycycline was found to achieve peak plasma levels of around 1 μg/mL. Doxycycline is a semi-synthetic tetracycline and remains the preferred tetracycline agent for most indications (81).

II. RESEARCH HYPOTHESIS AND OBJECTIVES

RESEARCH HYPOTHESIS

It was hypothesized that due to the known effects of osteopenia/osteoporosis in the bone tissue structure and metabolism, doxycycline, known to enhance osteoblast functionality in physiological conditions, could further assist on the improvement of the osteogenic activity in the established impaired conditions.

OBJECTIVES

1) To establish rat models representative of the human condition of osteopenia and osteoporosis;

2) To establish and characterize bone marrow-derived osteoblastic cultures from sham (control), osteopenic and osteoporotic animals, in the absence of exogenous osteogenic stimuli;

3) To address the effects of doxycycline in the proliferation and functional activity of established sham, osteopenic and osteoporotic-derived osteoblastic cultures.

III. MATERIALS AND METHODS

MATERIALS

All cell culture chemicals and supplies were acquired from Merck and Sigma Aldrich (St. Louis, MO) unless otherwise noted. All tissue culture flasks and plates were obtained from Corning (Corning, NY). Doxycycline was obtained from Sigma Aldrich.

METHODS

ANIMALS

Animals and Experimental Groups

This experimental study was performed under the authorization of Direção Geral de Alimentação e Veterinária and observed the technical standards of protection of experimental animals, according to the Portuguese (Decree No. 1005/92) and European (Directive 2010/63) legislations.

Nine nulliparous female Wistar rats, aged six weeks, were purchased from a certified vendor (Charles River laboratories, Barcelona) and housed in plastic cages, in a monitored environment, throughout the study period. Animals were given standard diet (4RF24 GLP, Mucedola®, Italy) and water, *ad libitum*. After a quarantine period of two weeks, rats were randomly assigned and housed together, in one of three groups: ovariectomized (OVX), sham (SHAM) and aged (AGE).

Ovariectomy was performed to mimic the human osteoporotic post-menopausal condition, while the osteopenic condition was established through the physiological evolution of the aging process. Methodological approach and specific time points for ovariectomy and animal euthanasia were carefully selected accordingly to established data from the literature (82). Available information supports a fast modification of bone parameters following ovariectomy – similar to those attained in human post-menopausal osteoporosis – and a slower process of bone modification with aging, similar to the one verified in osteopenic human adults (82).

Accordingly, animals of the OVX group were ovariectomized at 8 weeks of age and euthanized at 20 weeks of age. Animals from the SHAM and AGE groups were submitted to a sham surgical intervention at 8 weeks of age, in order to minimize the bias of the surgical intervention. Animals of the SHAM groups were euthanized at 20 weeks of age and animals of the AGE group, were euthanized at 48 weeks of age. A schematic representation of the experimental protocol is depicted in Figure 13 .

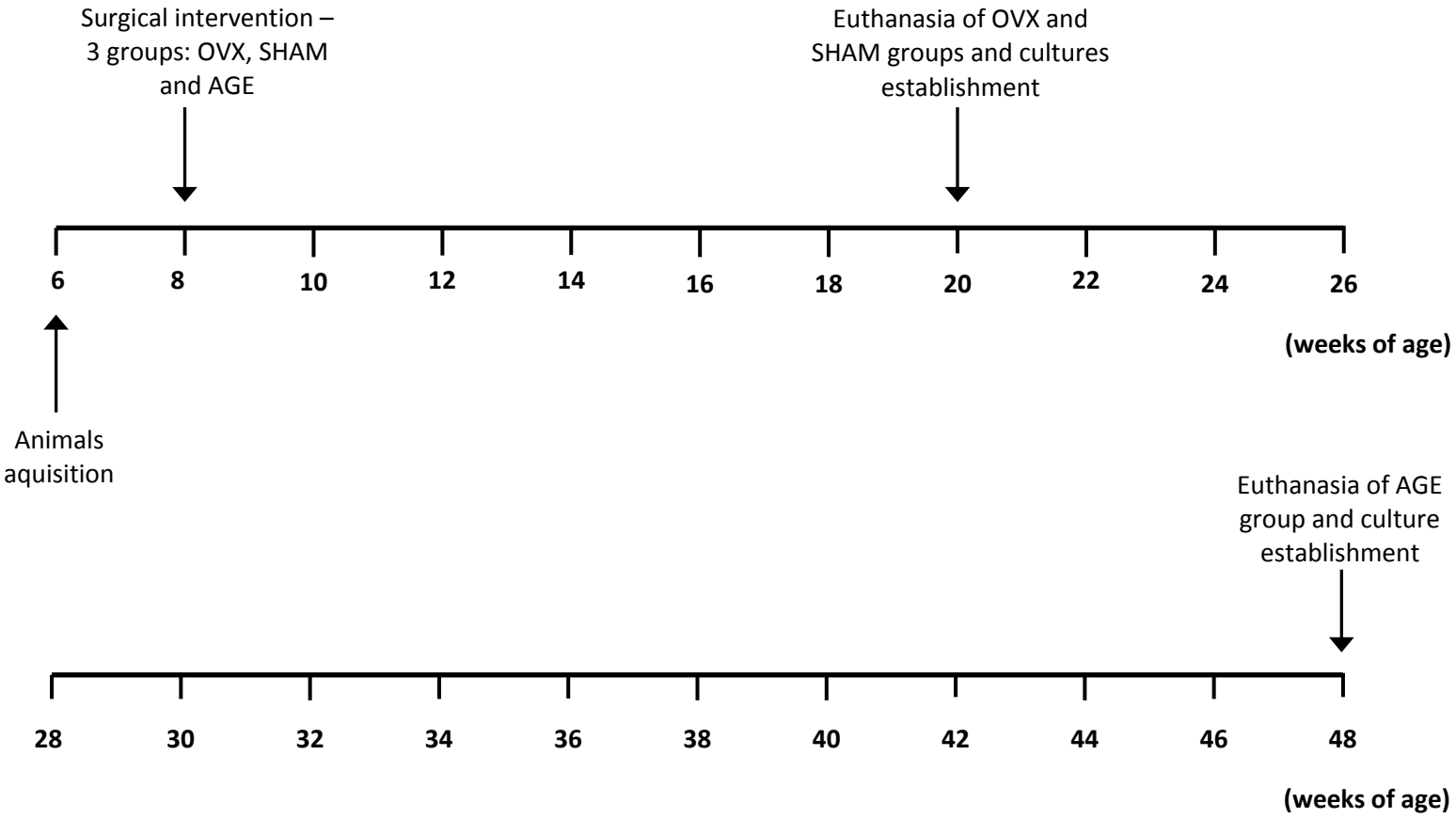


Figure 13 – Schematic representation of the experimental protocol.

Ovariectomy and Sham Surgical Procedure

Animals assigned to the OVX group were anesthetized by an intraperitoneal (IP) injection of xylazine (10mg/kg) and ketamine (90mg/kg). The abdominal cavity was assessed by a small midline dorsal skin incision, which allowed the blunt dissection of the connective tissue between the skin and the muscular layer of abdominal wall. Following, the muscular layers were opened half way down the side of the animal. Ovaries were identified and following pulled out through the incision. Two ligatures were placed, with absorbable 4-0 sutures: one in the caudal end, between the ovary and the uterine horn, and the other one in the cranial end of the ovary. Ovaries were following safely cut off and uterine horns pushed back into the abdominal cavity. Following inspection for abdominal hemorrhage, the abdominal muscle tissue was sutured with absorbable 4-0 sutures. Finally, the skin was sutured and animals were administered tramadol (10mg/Kg) IP, for post-operative analgesia.

The same procedure was performed to all animals assigned to the SHAM and AGE groups, with the exception of the placement of ligatures and ovaries removal. Along the experimental protocol the animal's weight was recorded monthly.

Assessment of the Bone Tissue Structure

In order to evaluate bone morphometric parameters, harvested tibias were submitted to microtomographic analysis (μCT). This is a non-destructive technique that allows the visualization and morphometric evaluation of microtomographic sections.

Proximal tibia sections were digitized with an X-ray source of 50 Kv and an intensity of 150 μA, with a voxel size of 12 μm. For each sample, a volume-of-interest (VOI) was defined within the trabecular component of the proximal tibia. VOI was delimited, 1 mm away from the growth plate, and with a 2 mm length on the metaphysis-diaphysis direction. 600 sections were acquired in each region.

The following variables were calculated:

- BV/TV: fraction of a given volume of interest (VOI, i.e. the Total Volume, TV) that is occupied by mineralized bone (Bone Volume, BV), expressed as %;
- Tb N: trabecular number, expressed as 1/mm;
- Tb Th: trabecular thickness, expressed as mm;

- Tb Sp: trabecular separation (or medular thickness), expressed as mm;
- Mean Density: mean voxel values of tissue within BV, expressed as units of HA density.

Different variables were calculated according to the methodological approaches described in the literature (83-85).

ESTABLISHMENT OF THE BONE-MARROW DERIVED OSTEOBLASTIC CULTURES

Cell cultures were obtained from bone marrow precursor cells of rat (*rattus norvegicus*) from Wistar rats, of the previously defined experimental groups. Animals were euthanized and preceded to the removal of their long bones from lower limbs – femurs and tibias – which were then placed in a decontamination solution: α-Minimal Essential Medium (α-MEM) supplemented with 25 µg/mL of fungizone (FUNGI) and 1000 IU/mL-1000 IU/mL of penicillin-streptomycin (PEST). After 20 minutes in this solution, bones were transferred to another solution with the same composition for another 20 minutes.

Once this time, epiphysis of all bones were removed and bone marrow cells were collected through perfusion of the spinal canal with culture medium: α-MEM supplemented with 10% fetal bovine serum (FBS), 2.5 µg/mL of FUNGI, 100 IU/mL-100 IU/ml of PEST and 50 µg/mL of ascorbic acid (AA). The obtained cell suspension was seeded in 6-well plates with 9.5 cm^2 area at a temperature of 37°C in a humidified atmosphere of 95% air and 5% CO_2 (incubation stove with controlled atmosphere). After 7 days, the medium was changed to remove the non-adherent cells and then replaced twice weekly. Cultures were regularly observed by phase contrast microscopy.

Cultures were maintained up to a confluence of approximately 80% – 15 days of culture. At this day cells were enzymatically released by trypsinization (trypsin 0.04%). Cells in suspension were counted in a hematocitometer (Nihon Kohden, MEK-5103K) and seeded at a density of $2x10^4$ cells per square cm in 96-well plates (0.32 cm^2 area). At adequate experimental settings, cultures were grown in the presence of doxycycline $1\mu g.ml^{-1}$. The culture medium was replaced twice weekly. Cells were maintained this way for 14 days.

Cell cultures were characterized for viability, proliferation and differentiation event.

CHARACTERIZATION OF CELL CULTURES

Cultures characterization – morphology, proliferation, viability and cell activity – was conducted through observation by optical microscopy and by histochemical and biochemical methods.

Optical Microscopy

Cell cultures monitoring was conducted periodically using phase contrast optical microscopy for the evaluation of cell morphology and proliferation.

Cell Proliferation Assay (DNA Content)

Cell proliferation was estimated by the DNA content at specific time points. DNA content was analysed by the PicoGreen DNA quantification assay (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes Inc., Eugene), according to manufacturer's instructions. Cultures were treated with Triton X-100 (0.1%) (Sigma) and fluorescence was measured on an Elisa reader (Synergy HT, Biotek) at wavelengths of 480 and 520 nm, excitation and emission respectively, and corrected for fluorescence of reagent blanks. The amount of DNA was calculated by extrapolating a standard curve obtained by running the assay with the given DNA standard.

Metabolic Activity Assay (MTT Assay)

Metabolic activity of the cells was assessed by the MTT assay (86,87), which is based on the reduction of tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) by viable or metabolically active cells. As a result, it occurs the formation of purple crystals, designated formazan crystals. Following, the spectophotometric determination of the colored product is conducted.

The MTT analysis was performed at specific time points. On the selected day a MTT solution (5mg/ml) was added to each culture well and the cells were incubated for 4 hours in the conditions used during cell culture (37°C, humidified atmosphere of 95% air and 5% CO_2). After the incubation period, culture medium was removed, dimethylsulphoxide (DMSO) was added to each well and plates were shaken to dissolve the formazan crystals. The absorbance was measured in an ELISA reader (Biotek, model Synergy HT) at 550nm.

Apoptosis Assay

Apoptosis was estimated by the Caspase-3 activity at specific time points. Caspase-3 activity was analysed by the Caspase-3 assay (EnzChek® Caspase-3 Assay Kit #2), according to manufacturer's instructions. Cultures were treated with Triton X-100 (0.1%) (Sigma) to obtain cell lysates and following, Caspase-3 activity was evaluated by fluorescence on an Elisa reader (Synergy HT, Biotek) at wavelengths of 480 and 520 nm, excitation and emission respectively, and corrected for fluorescence of reagent blanks. The amount of reduced substrate was calculated by extrapolating a standard curve obtained from Rhodamine 110 reference standard.

Assessment of Cell Morphology

Cell cultures morphology was characterized by confocal laser scanning microscopy (CLSM) following staining of cytoskeleton and nucleus counterstaining. For CLSM assessment, cell cultures were fixed in 3.7% paraformaldehyde during 15 minutes and permeabilized with Triton X-100 (0.1%). Then cells were incubated with albumin (10 $mg.ml^{-1}$) to reduce non-specific staining. Cell cytoskeleton filamentous actin (F-actin) was visualized treating the cells with Alexa Fluor 488®-conjugated phalloidin (1:20 dilution in PBS for 20 minutes) and counterstained with propidium iodide (1 $\mu g.ml^{-1}$ for 10 min) for cell nuclei labeling. Labeled cultures were mounted in Vectashield® and examined with a Leica SP2 AOBS (Leica Microsystems) microscopy.

Total Protein Content

The total protein content was determined using the Lowry method (88,89). This is based on the reaction of Folin-Ciocalteau reagent with aromatic amino acids, and there's the formation of a colored product, which is measured by spectrophotometry.

After removing the culture medium, cells were washed twice with PBS buffer ("Dulbeco's Phosphate Buffered Saline," Sigma D-1480), pH 7.4. It was added 100µL of Triton X-100 (0.1%) to each well and plates were placed in an incubator at 37°C for one hour. Once this time, samples were collected into eppendorf tubes, make up to volume of 300µl with NaOH 0.1 M and added to each sample 1.5 ml of an alkaline solution of copper. Allowed to act for 10 minutes, phenol reagent was added and the plate was placed in the dark for one hour. Once this time, absorbance was read in a spectrophotometer (Jenway, model 6300) at 750nm. Absorbance was compared with values obtained for a serie of standards of bovine serum albumin, made from a solution of 0.5 mg/mL in NaOH 0.1.

Results were expressed in µg of total protein in the sample per square centimeter ($\mu g/cm^2$).

Alkaline Phosphatase Activity

The alkaline phosphatase (ALP) activity was assessed by spectrophotometric determinination of the product obtained by hydrolysis of p-nitrophenylphosphate disodium (pNPP), upon reaction with ALP (p-nitrophenol).

The culture medium was removed and cells were washed twice with PBS buffer, pH 7.4. It was added 100µL of Triton X-100 (0.1%) to each well to induce cell lysis. Thereafter, the substrate was prepared, 80µl were placed in each well and plate was incubated at 37°C for 30 minutes.

Results were expressed as nanomoles of p-nitrophenol produced per minute per microgram of protein (nmol/min.µg protein) as compared with respective calibration straight line.

Histochemical Methods

Cell cultures were fixed at specific time points for further performance of histochemical techniques. The culture medium was removed and cells were washed with PBS at 37°C, pH 7.4. Cells were fixed with glutaraldehyde 1.5% for ten minutes. Thereafter, was added sodium cacodylate buffer at 0.14 M and samples were preserved at 4°C.

Alkaline Phosphatase Staining

Cell cultures were stained immunohistochemically to identify ALP activity by a method based on the hydrolysis of sodium naphthyl phosphate by ALP, and precipitation of phosphate liberated by reaction with a diazonium salt (Fast Blue RR). Hence, originates a colored product that, depending on amount of enzyme, may be yellow, brown or black.

Cultures were placed in the dark for one hour, after fixation with buffer Tris 0.1 M, pH 10, with 2 mg/ml of sodium naphthyl phosphate and 2 mg/ml of Fast Blue RR. After the incubation, samples were washed with distilled water and allowed to dry under ambient conditions.

Cultures were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

Collagen Staining

Collagen assay is based on the binding of a dye Sirius red F3BA (BDH, UK) – with the triple helical collagen fibril.

The staining of the fixed cells was performed with 0.1% siriusred F3BA in saturated picric acid at room temperature, during 1 hour, under mild shaking. Afterward, the dye solution was removed by suction and the stained cell layers intensively washed with 0.01 N hydrochloric acid to remove all non-bound dye.

Gene Expression Analysis

RT-PCR analysis was done in the three established cultures at specific time points. These were evaluated for the expression of ALP, bone morphogenic protein-2 (BMP-2), Col I, OPN, OC, OPG, PPARγ, CFD and AP-2. Total RNA was extracted using the NucleoSpin® RNA II Kit (Macherey-Nagel) according to the manufacturer's instructions. The concentration and purity of total RNA in each sample were assessed by UV spectrophotometry at 260 nm and by calculating the A260nm/A280nm ratio, respectively. RT-PCR was done using the Titan One Tube RT-PCR system (Roche® Applied Science), according to the manufacturer's instructions, for 28 cycles. RT reaction mixtures consisted of extracted RNA, Titan RT-PCR buffer, dithiothreitol (DTT), deoxynucleoside triphosphate (dNTP), primers for each tested gene, avian myeloblastosis virus RT (AMV-RT) and water, in a total volume of 25 µl. Total RNA was reverse transcribed with cDNA (30 minutes at 50ºC), which was then amplified with recombinant Taq-DNA polymerase at different annealing temperatures. For all the genes, the annealing temperature was 60ºC. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide and semi-quantitatively assessed by densitometry with Image J® software.

STATISTICAL ANALYSIS

Data presented in this work are the result of three separate experiments performed in cell cultures established from different animals. For biochemical data (MTT assay, total protein content and ALP activity) each point represents the mean ± standard error of 6 independent assays. Statistical analysis was done by one-way analysis of variance (ANOVA). P values ≤ 0.05 were considered significant.

IV. RESULTS

ASSESSMENT OF THE BONE TISSUE STRUCTURE

The bone morphometric parameters were assessed by µCT analysis and the results are in Table 1. The BV/TV and Tb N values decreased in the osteopenic group and were even lower in osteoporotic group. The Tb Th and Tb Sp were found not to differ significantly between groups despite that Mean Density was significant reduced in osteopenic and osteoporotic groups, as comparing to sham.

Table 1 – µCT values from Sham, Osteopenic and Osteoporotic groups.

* – significantly different from control ($p < 0.05$).

	Sham	**Osteopenic**	**Osteoporotic**
BV/TV (%)	54.33 ± 6.3	42 ± 4.78 *	17.78 ± 3.6 *
Tb N (1/mm)	6.073 ± 0.54	5.329 ± 0.68 *	1.892 ± 0.41 *
Tb Th (mm)	0.0897 ± 0.012	0.1065 ± 0.009	0.0917 ± 0.008
Tb Sp (mm)	0.0998 ± 0.018	0.1278 ± 0.012	0.5452 + 0.014
Mean Density (mg HA/ccm)	883.5004 ± 57.302	846.3288 ± 32.795 *	836.1752 ± 44.784 *

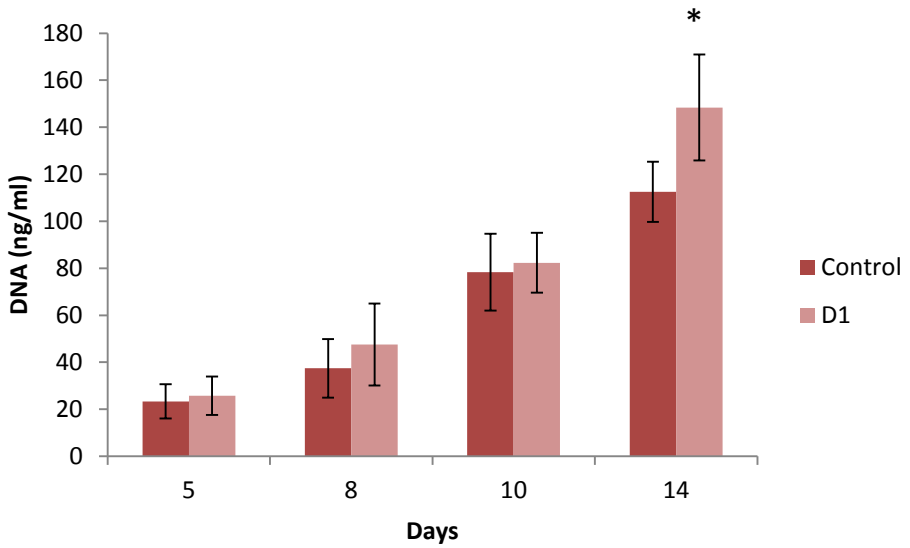
ESTABLISHMENT OF BONE MARROW-DERIVED OSTEOBLASTIC CULTURES FROM SHAM (CONTROL) ANIMALS

In the first part of this study, the principal aim was to establish a bone marrow-derived osteoblastic culture from sham animals in two different conditions – in the absence and in the presence of doxycycline at 1 µg/ml. Cultures established in the absence of doxycycline were referred as ‘Control’; and cultures grown with doxycycline were termed as ‘D1’.

Cells were cultured in control medium and the first subcultures were evaluated for cell morphology, cell viability/proliferation and osteoblastic differentiation throughout the 14 days of culture time.

CELL PROLIFERATION

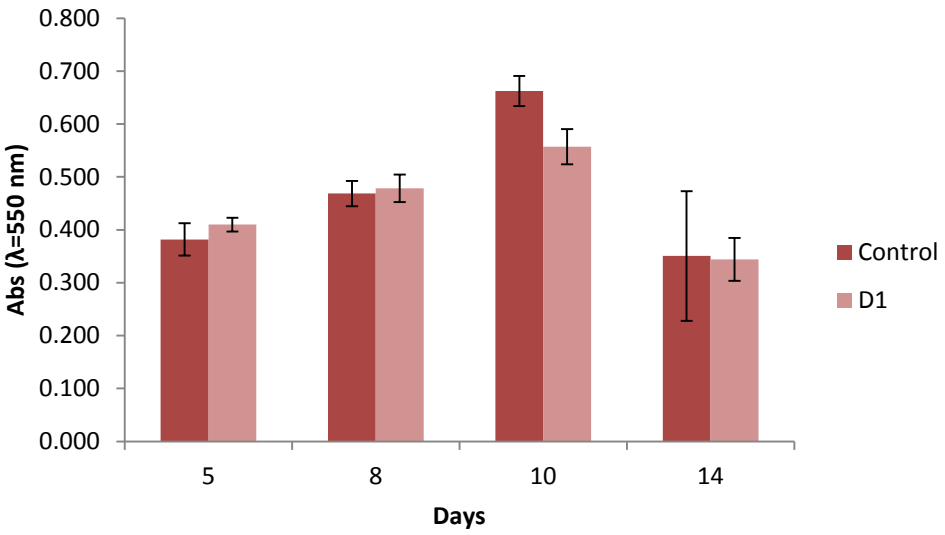
Graphic 1 shows the results of cell proliferation that was assessed by the DNA content. Control cell culture established from sham animals increased the proliferation rate during the time of culture. The culture with doxycycline had a similar behavior – increase in cell proliferation from day 5 to day 14 of culture. The addition of doxycycline revealed higher values in the proliferation rate.



Graphic 1 – Cell proliferation of sham rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
* – significantly different from control ($p < 0.05$).

METABOLIC ACTIVITY

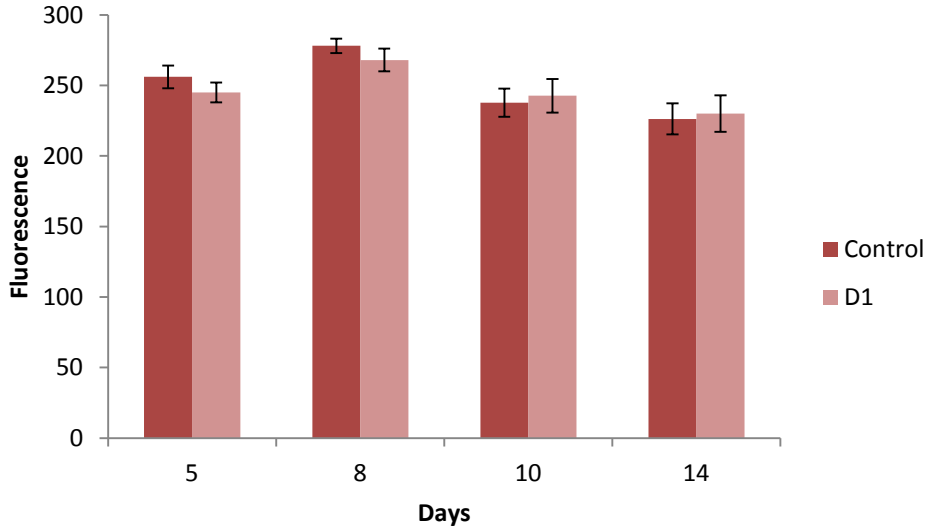
Metabolic activity was evaluated by the MTT assay (Graphic 2). Cultures established from sham animals presented an increase in MTT reduction values till day 10 and suffered a decrease of almost 50% at day 14. The cultures with doxycycline showed a similar behavior – MTT values increased from day 5 to day 10 and decreased at day 14. The metabolic activity in the culture with doxycycline is slightly higher than the one in the control culture in the first days of culture, but this pattern is reverted in the last days of culture.



Graphic 2 – Metabolic activity of sham rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

APOPTOSIS

Apoptosis was assessed by the Caspase-3 assay and is represented in Graphic 3. The apoptosis is broadly constant during the time of culture. The values between the control culture and the one with doxycycline are very similar with no significant differences between experimental conditions.



Graphic 3 – Apoptosis of sham rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

CELL MORPHOLOGY

CLSM was the method used for the assessment of cell morphology and the acquired images are shown in Figure 14. At day 8, cells of the control culture exhibited expanded cytoplasm and an adequate nuclear organization. Besides that, several cell to cell contact were established. The cells proliferated adequately and, at this time point, a large area of the culture surface was already covered by cells. The cellular behavior of cultures with doxyxycline was similar.

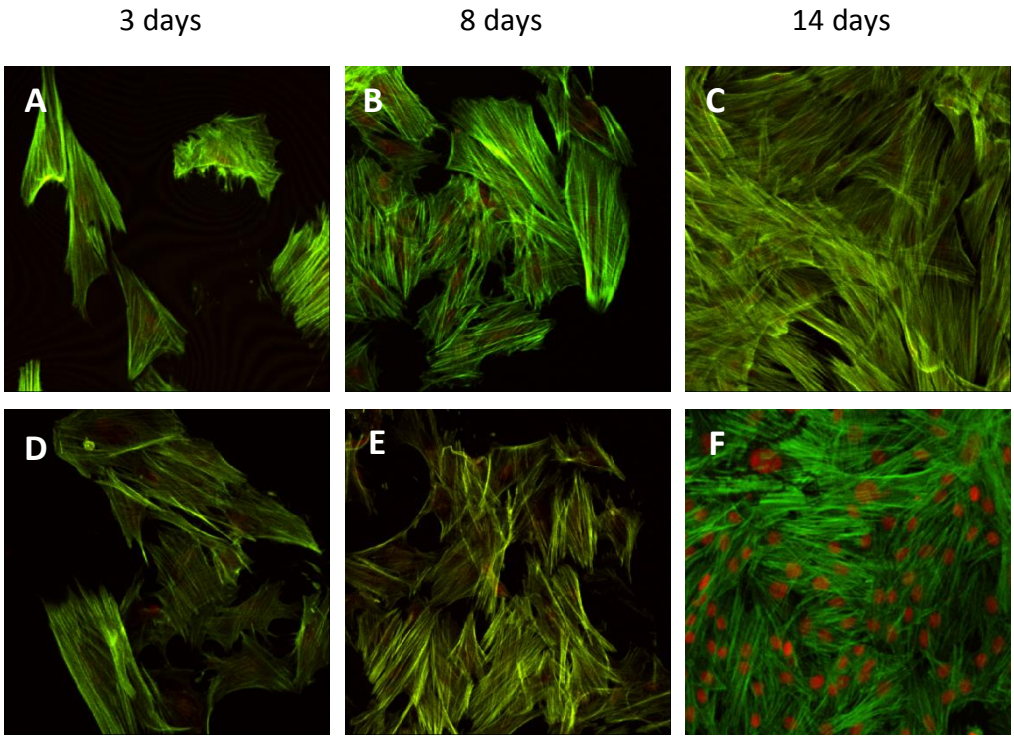
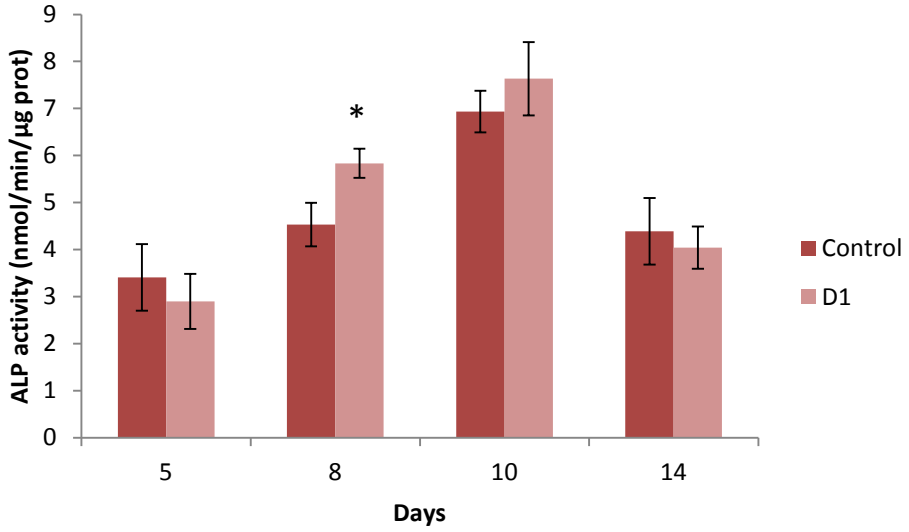


Figure 14 – CLSM imaging of sham rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
Cytoskeleton was stained in green and nucleus counterstained in red. A to C corresponds to control cultures, and D to F corresponds to cultures with doxycycline. Magnification 400x.

ALKALINE PHOSPHATASE ACTIVITY

Results of ALP activity were normalized by total protein content and are show in Graphic 4. In the culture established from sham animals, alkaline phosphatase activity increased from day 5 to day 10 and suffered a decrease at day 14. The culture established in the presence of doxycycline showed a similar behavior – increased in the first days of culture and decreased at day 14. Comparing the two cultures, the ALP activity is higher in doxycycline culture at eighth and tenth days of culture.



Graphic 4 – ALP activity of sham rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
* – significantly different from control ($p < 0.05$).

ALKALINE PHOSPHATASE STAINIG

Representative figures of ALP staining are shown in Figure 15. Both control and doxycycline cultures presented a similar behavior concerning the production and distribution of ALP. Grown cells show a homogeneous ALP staining at day 5, in which the first cell clusters are detectable. This organization is easier identified at days 8 and 10, in which aggregates of cells have a more intensive staining – dark brown/black staining. This demonstrates an increase in ALP activity over time culture.

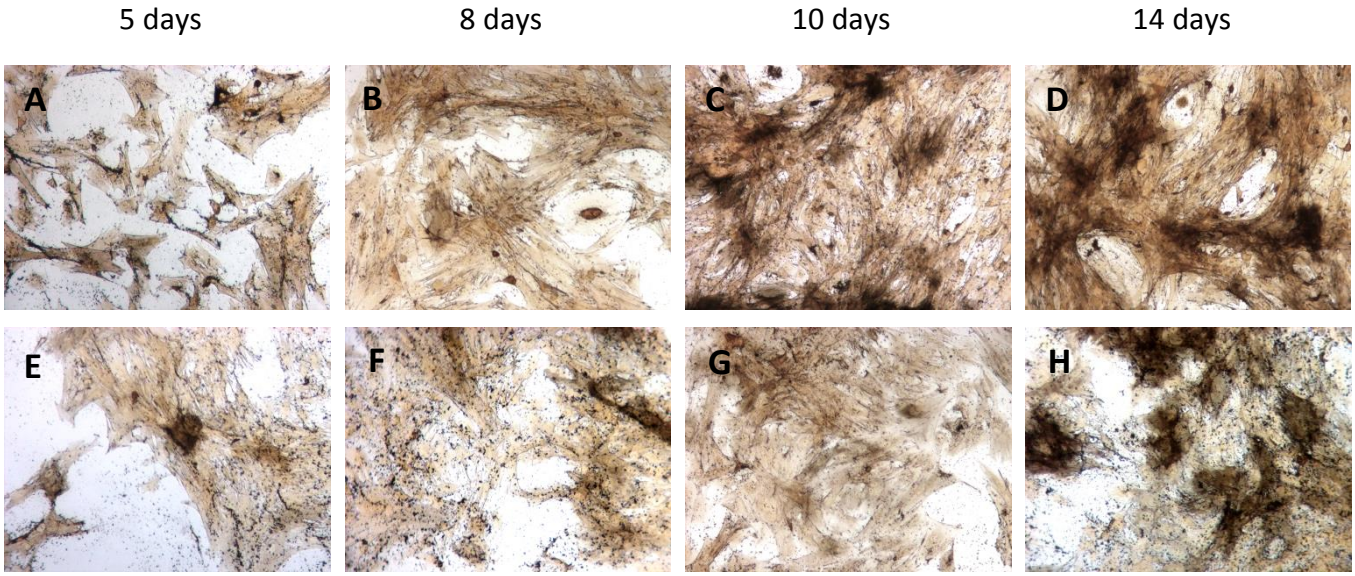


Figure 15 – ALP staining of sham rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

A to D corresponds to control cultures, and E to H corresponds to cultures with doxycycline. Magnification 100x.

COLLAGEN STAINING

Figure 16 shows representative images of collagen staining in cultures established from sham animals. In control culture is evident the formation of nodular structures since day 5 of culture which increased till day 14. Both cultures revealed an increase in the color staining in later time points, days 11 and 14.

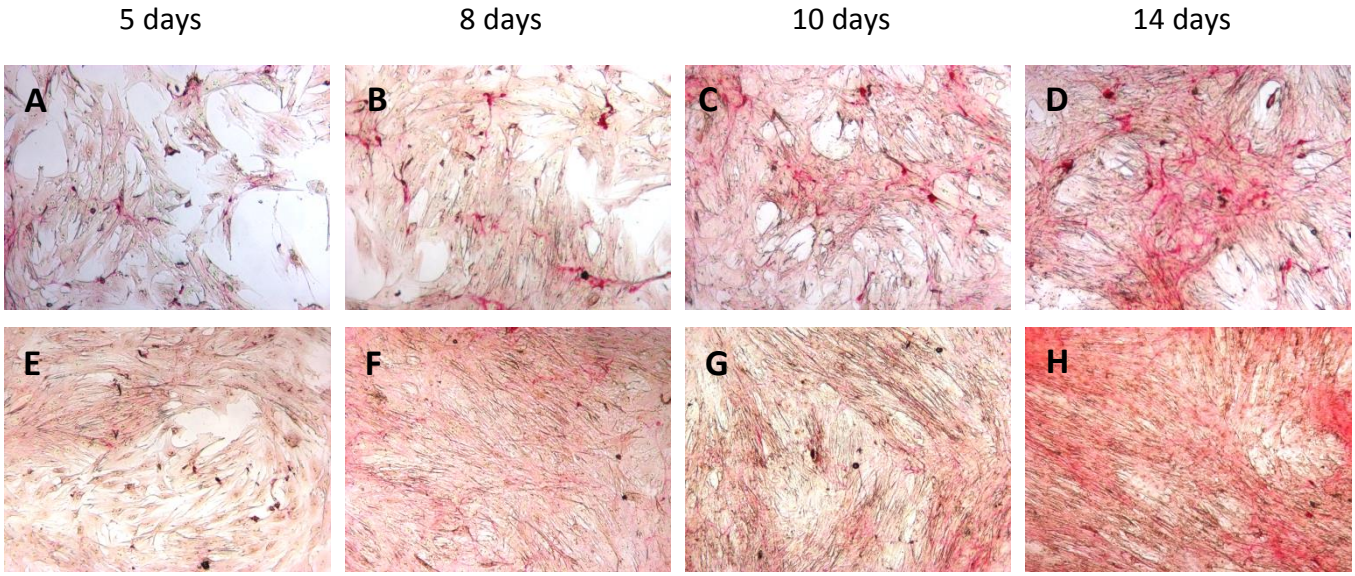
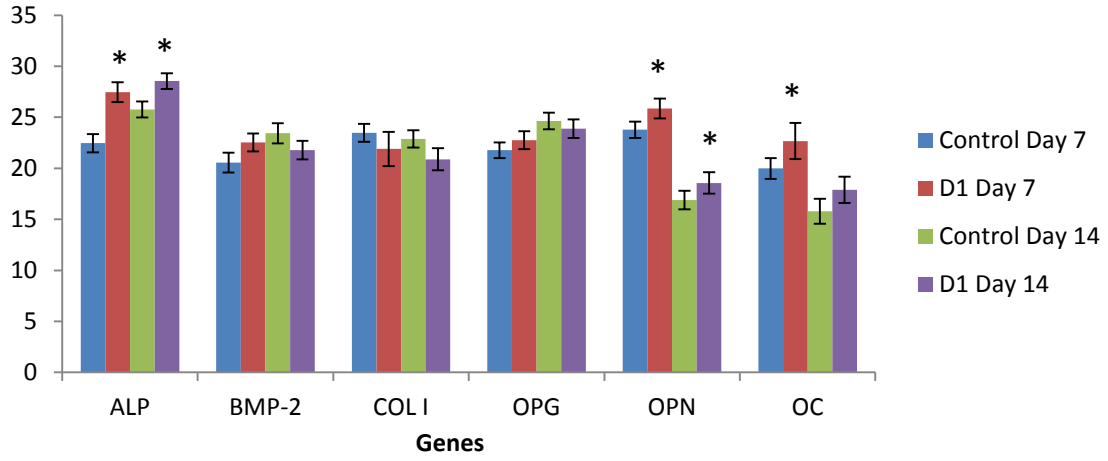


Figure 16 – Collagen staining of sham rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
A to D corresponds to control cultures, and E to H corresponds to cultures with doxycycline. Magnification 100x.

EXPRESSION OF OSTEOGENIC-RELATED MARKERS

RT-PCR analysis showed that both experimental conditions – sham control cultures and with doxycycline – expressed high levels of significant osteogenic markers. Results are shown in Graphic 5.

At day 7, control cultures expressed similar levels of ALP, BMP-2, Col I, OPG and OPN, while the levels of OC were lower. Cultures with doxycycline at this time point expressed higher levels of all of these markers except Col I that showed reduced values. At day 14, the expression of BMP-2, Col I and OPG slightly increased. On the other hand, the expression levels of OPN and OC decreased. In general, cultures with doxycycline at day 14 increased the expression of osteogenic markers.



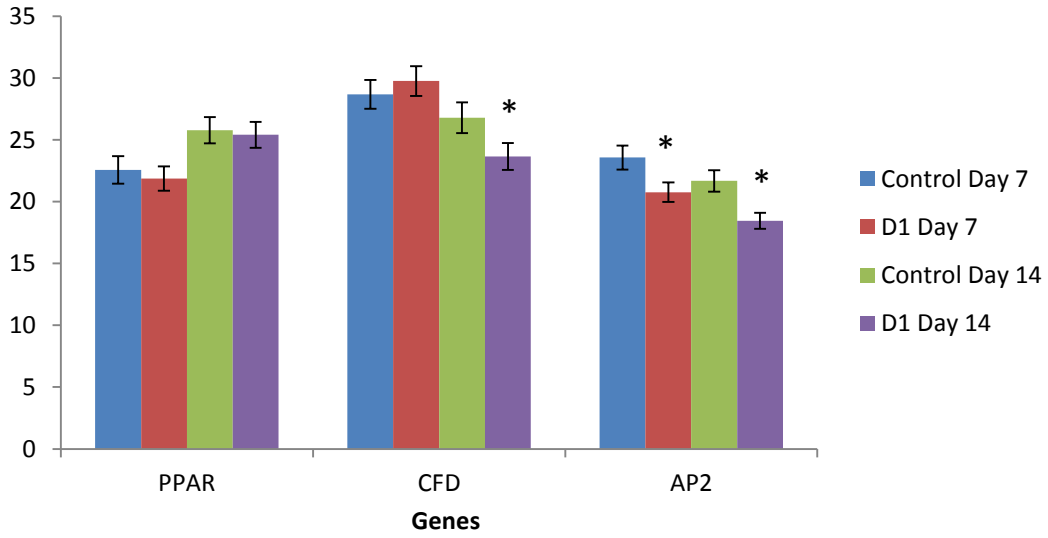
Graphic 5 – RT-PCR gene expression of ALP, BMP-2, Col I, OPG,OPN and OC, in sham rat bone marrow-derived cell cultures established at days 7 and 14, in the absence and in the presence of doxycycline.

* – significantly different from control ($p< 0.05$).

EXPRESSION OF ADIPOGENIC-RELATED MARKERS

RT-PCR analysis of adipogenic markers in cultures established from sham animals is shown in Graphic 6.

At day 7, the expression levels of AP-2 decreased in cultures with doxycycline. Also in the presence of doxycycline, CFD and AP-2 expression decreased, at day 14.



Graphic 6 – RT-PCR gene expression of PPARγ, CFD and AP-2, in sham rat bone marrow-derived cell cultures established at days 7 and 14, in the absence and in the presence of doxycycline

* – significantly different from control ($p < 0.05$).

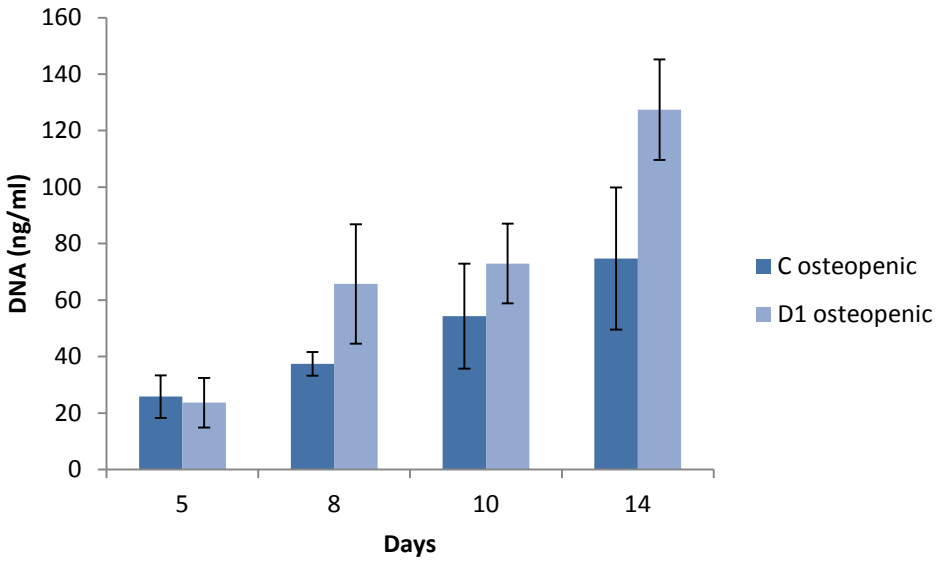
ESTABLISHMENT OF BONE MARROW-DERIVED OSTEOBLASTIC CULTURES FROM OSTEOPENIC ANIMALS

In the second part of this study, the principal aim was to establish a bone marrow-derived osteoblastic culture from osteopenic animals in two different conditions – in the absence and in the presence of doxycycline at 1 µg/ml. Cultures established in the absence of doxycycline were referred as 'C osteopenic'; and cultures with doxycycline were termed as 'D1 osteopenic'.

Cells were cultured in control medium and the first subcultures were evaluated for cell morphology, cell viability/proliferation and osteoblastic differentiation throughout the 14 days of culture time.

CELL PROLIFERATION

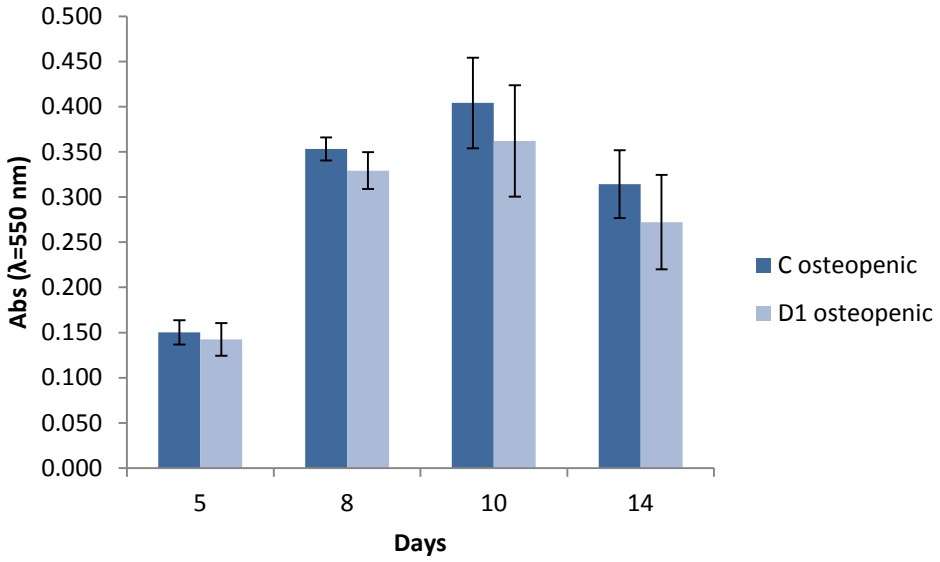
Cell proliferation was assessed by the DNA content and the results are in Graphic 7. Control cell culture from osteopenic animals showed an increasing proliferation throughout the culture time. In the culture with doxycycline, cell proliferation started with a lower value that is enhanced in almost 50% at day 8. Cell proliferation continued to increase and reached the highest value at day 14.



Graphic 7 – Cell proliferation of osteopenic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

METABOLIC ACTIVITY

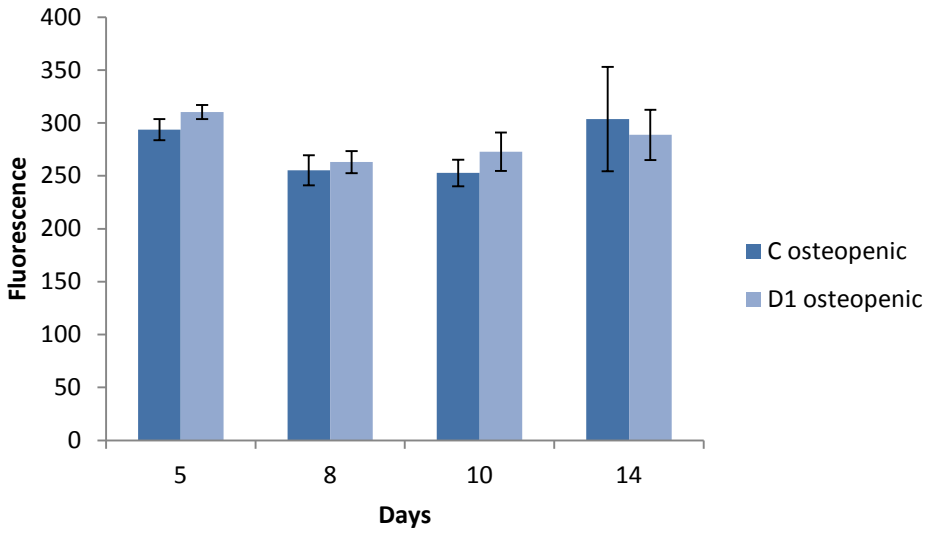
Metabolic activity was evaluated by the MTT assay (Graphic 8). Cultures established from osteopenic animals without introduction of any osteogenic inducers presented an increasing until day 10 and then suffered a slight decline. Cultures with doxycycline had a similar behavior – the MTT value increased from the first day of culture till day 10 and then decreased at day 14. The MTT reduction values were similar between the two experimental conditions.



Graphic 8 – Metabolic activity of osteopenic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

APOPTOSIS

Apoptosis was assessed by the Caspase-3 assay and is represented in Graphic 9. The apoptosis levels were kept broadly constant throughout the culture period. The values of apoptosis were similar in the two experimental conditions.



Graphic 9 – Apoptosis of osteopenic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

CELL MORPHOLOGY

CLSM was the method used for the assessment of cell morphology and the acquired images are shown in Figure 17. At early time points cells reported a characteristic osteoblastic morphology and evident cell to cell contacts. Cells proliferated actively and attended cell multilayer organization at later time points. No significant differences were found in cell morphology in control cultures and cultures with doxycycline.

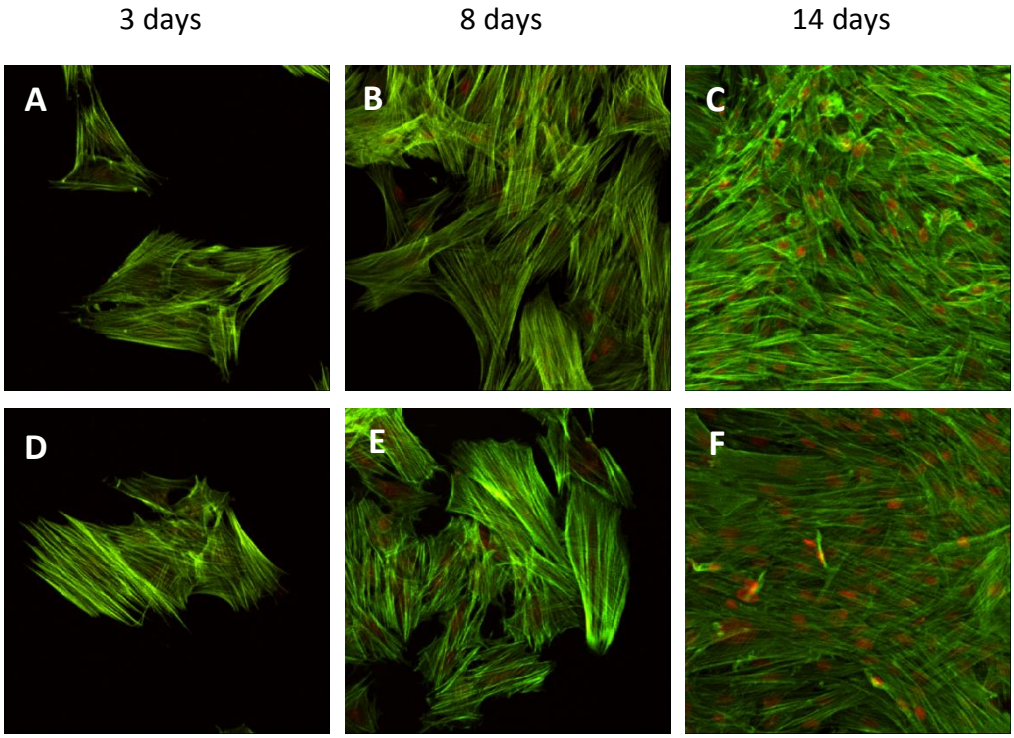
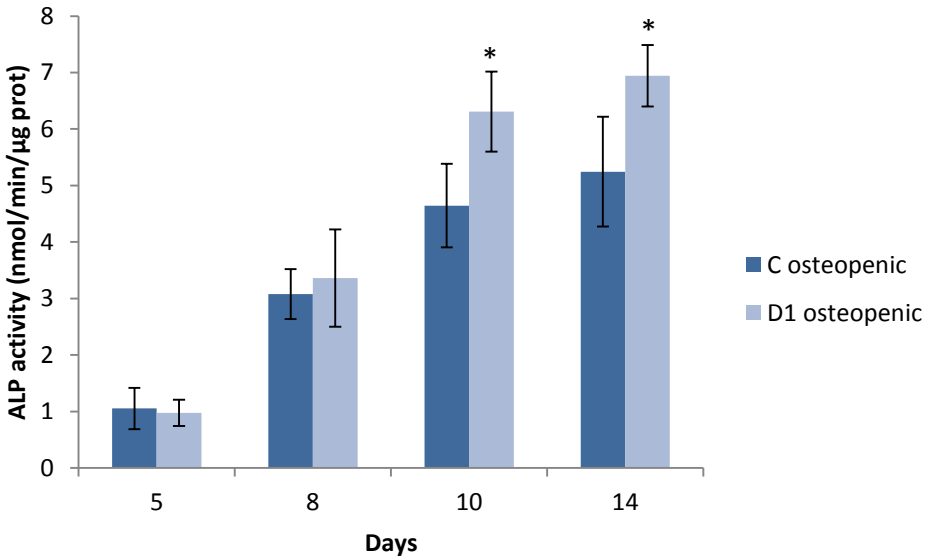


Figure 17 – CLSM imaging of osteopenic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
Cytoskeleton was stained in green and nucleus counterstained in red. A to C corresponds to control cultures, and D to F corresponds to cultures with doxycycline. Magnification 400x.

ALKALINE PHOSPHATASE ACTIVITY

Results of ALP activity were normalized by total protein content and are show in Graphic 10. In the control culture established from osteopenic animals, ALP activity showed an increase over the time of culture. The culture established in the presence of doxycycline demonstrates a similar behavior, increasing the ALP activity from the first day till the last day of culture. Comparing the two experimental conditions, the ALP activity in the culture with doxycycline was significantly higher since the eighth day of culture.



Graphic 10 – ALP activity of osteopenic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

* – significantly different from control ($p < 0.05$).

ALKALINE PHOSPHATASE STAINIG

Representative images of ALP staining are shown in Figure 18. It was verified an increased intensity for the two experimental conditions throughout the culture time. In both conditions the nodular aggregates were already visible at day 5 and increased over time, covering the vast majority of the culture. In later time points, the staining increased acquiring a dark brown/black staining, which is detectable in both conditions. This is in agreement with the ALP activity values showed above.

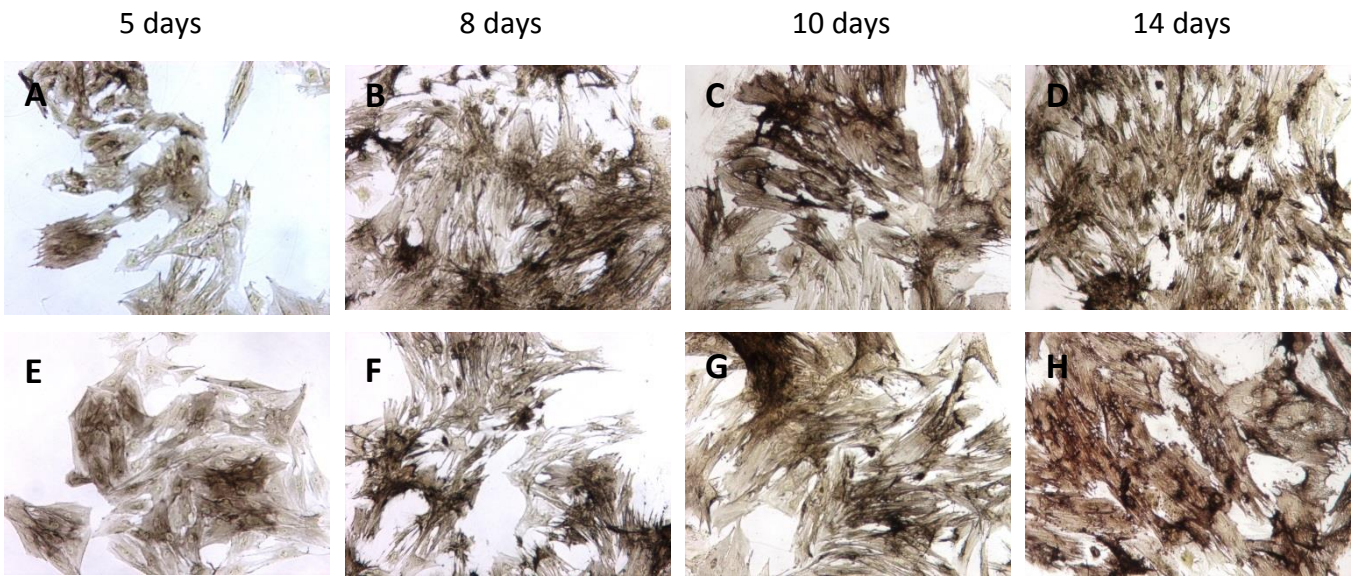


Figure 18 – ALP staining of osteopenic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
A to D corresponds to control cultures, and E to H corresponds to cultures with doxycycline. Magnification 100x.

COLLAGEN STAINING

Representative images of collagen staining in osteopenic cultures with and without doxycycline are shown in Figure 19. In both conditions it's verified the formation of nodular structures that increased over the time culture. The intensity of the staining also increases and in later time points, days 11 and 14, the pink is more accentuated.

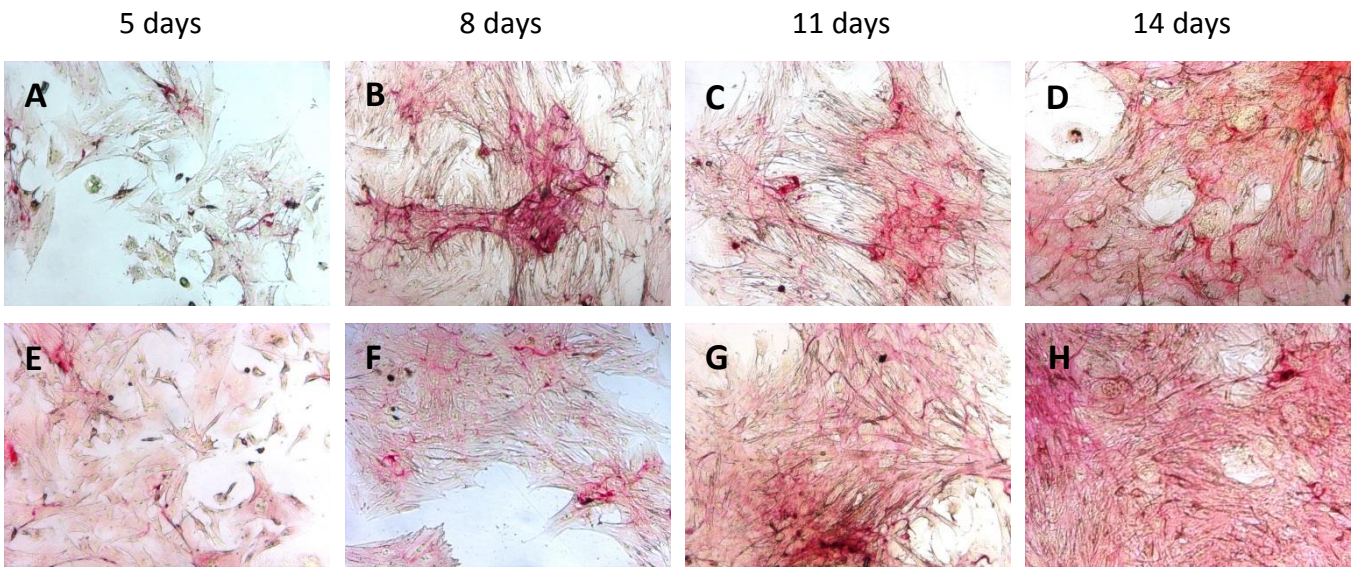
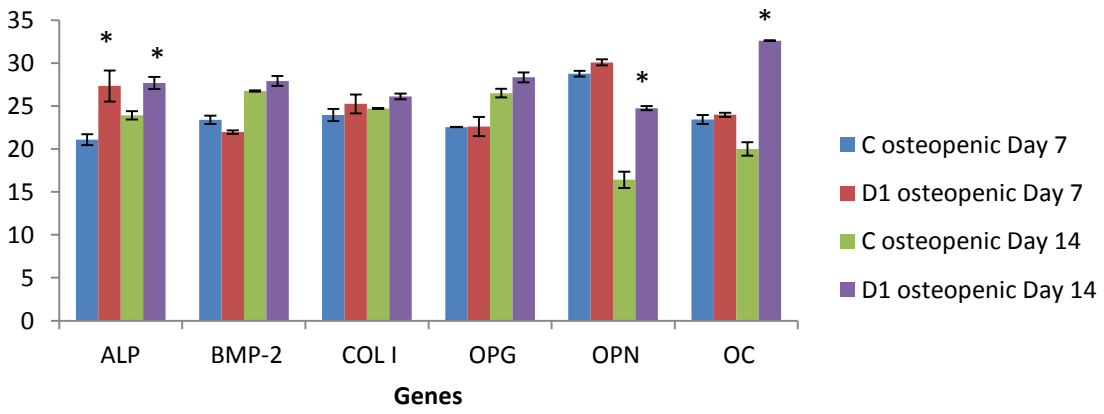


Figure 19 – Collagen staining of osteopenic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
A to D corresponds to control cultures, and E to H corresponds to cultures with doxycycline. Magnification 100x.

EXPRESSION OF OSTEOGENIC-RELATED MARKERS

RT-PCR analysis showed that both experimental conditions – osteopenic control cultures and with doxycycline – expressed high levels of significant osteogenic markers. Results are shown in Graphic 11.

At day 7, cultures with doxycycline expressed higher levels of ALP, Col I and OPN, comparing with the control culture. At day 14, control cultures expressed reduced levels of OPN and OC, while the expression of ALP, BMP-2, Col I and OPG was elevated. At the same time point, cultures with doxycycline expressed higher levels of these genes.



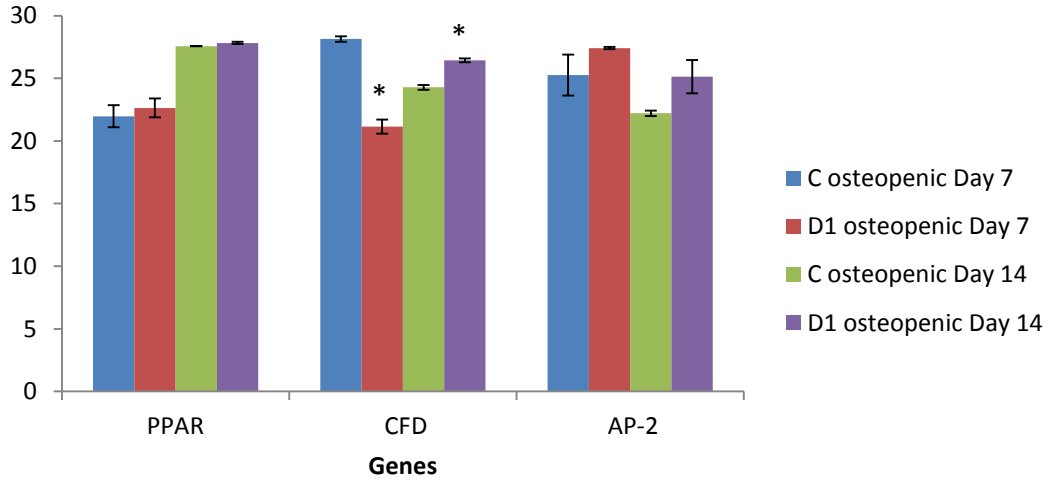
Graphic 11 – RT-PCR gene expression of ALP, BMP-2, Col I, OPG, OPN and OC, in osteopenic rat bone marrow-derived cell cultures established at days 7 and 14, in the absence and in the presence of doxycycline.

* – significantly different from control ($p < 0.05$).

EXPRESSION OF ADIPOGENIC-RELATED MARKERS

RT-PCR analysis of adipogenic markers is shown in Graphic 12.

At day 7, the expression levels of CFD decreased in cultures with doxycycline. On the other hand, at day 14, in cultures with doxycycline the expression of CFD increased in comparison with the control culture.



Graphic 12 – RT-PCR gene expression of PPARγ, CFD and AP-2, in osteopenic rat bone marrow-derived cell cultures established at days 7 and 14, in the absence and in the presence of doxycycline.

* – significantly different from control ($p < 0.05$).

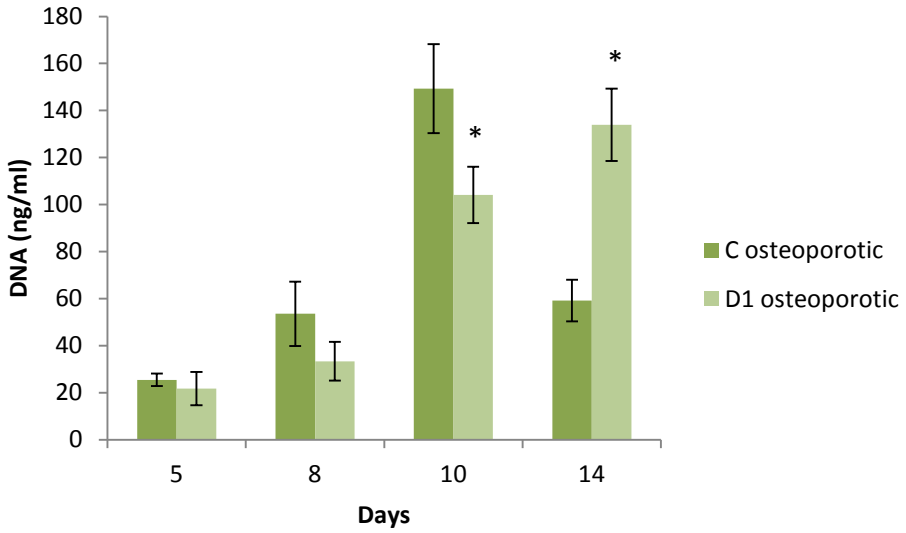
ESTABLISHMENT OF BONE MARROW-DERIVED OSTEOBLASTIC CULTURES FROM OSTEOPOROTIC ANIMALS

In the third part of this study, the principal aim was to establish a bone marrow-derived osteoblastic culture from osteoporotic animals in two different conditions – in the absence and in the presence of doxycycline at 1 µg/ml. Cultures established in the absence of doxycycline were referred as 'C osteoporotic'; and cultures with doxycycline were termed as 'D1 osteoporotic'.

Cells were cultured in control medium and the first subcultures were evaluated for cell morphology, cell viability/proliferation and osteoblastic differentiation throughout the 14 days of culture time.

CELL PROLIFERATION

The DNA content was the method used to assess the cell proliferation of the established cultures (Graphic 13). In the control culture from osteoporotic animals, cell proliferation increased till the tenth day of culture, being the increase from day 8 to day 10 very accentuated. Then cell proliferation decreased greatly at day 14. The culture with doxycycline didn't have a similar behavior, increasing in a more regular way over the time of culture. The DNA values are lower in the doxycycline culture than in the control culture, except for the fourteenth day of culture.

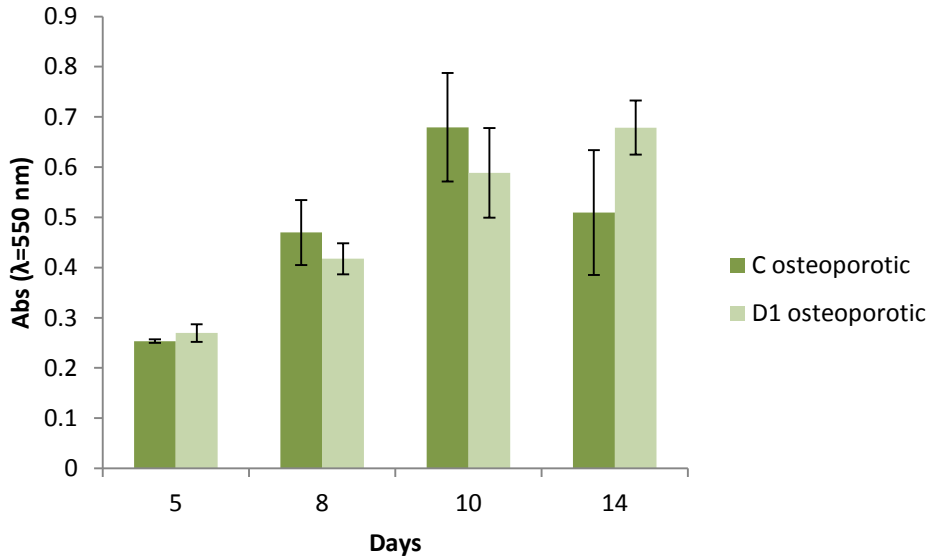


Graphic 13 – Cell proliferation of osteoporotic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

* – significantly different from control ($p < 0.05$).

METABOLIC ACTIVITY

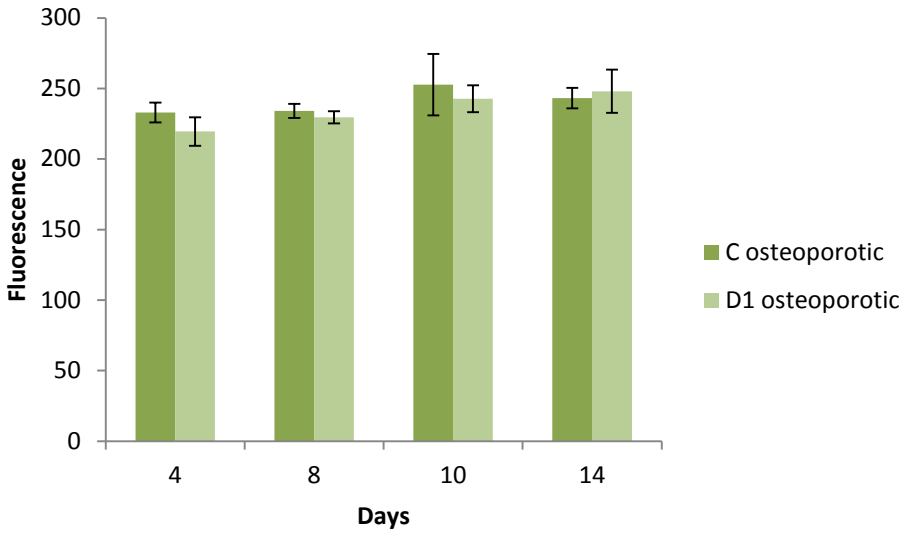
Metabolic activity was evaluated by the MTT assay (Graphic 14). Cultures established from osteoporotic animals without introduction of any osteogenic inducers presented an increasing from the first day till day 10 and then showed a slight decrease in the last day of culture. The time-course growth pattern of cultures with doxycycline was a little bit different – MTT reduction values increased over the 14 days of culture.



Graphic 14 – Metabolic activity of osteoporotic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

APOPTOSIS

Apoptosis was assessed by the Caspase-3 assay and is represented in Graphic 15. The apoptosis is constant throughout the culture time and there are no significant differences between the two experimental conditions.



Graphic 15 – Apoptosis of osteoporotic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.

CELL MORPHOLOGY

CLSM was the method used for the assessment of cell morphology and the acquired images are shown in Figure 20. In control cultures, cells were elongated and the actin fibers were with an intense staining. Cell to cell contacts were established and, at day 8, an organized flattened sheet of continuous cell layers were verified. The cultures with doxycycline presented a similar cellular behavior.

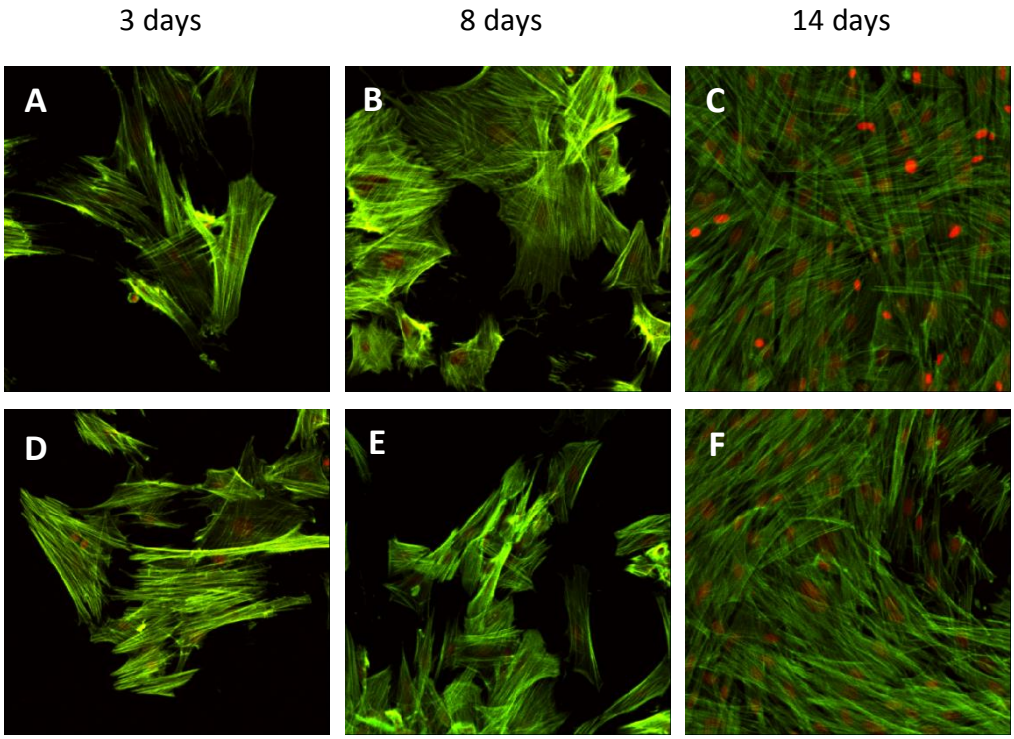
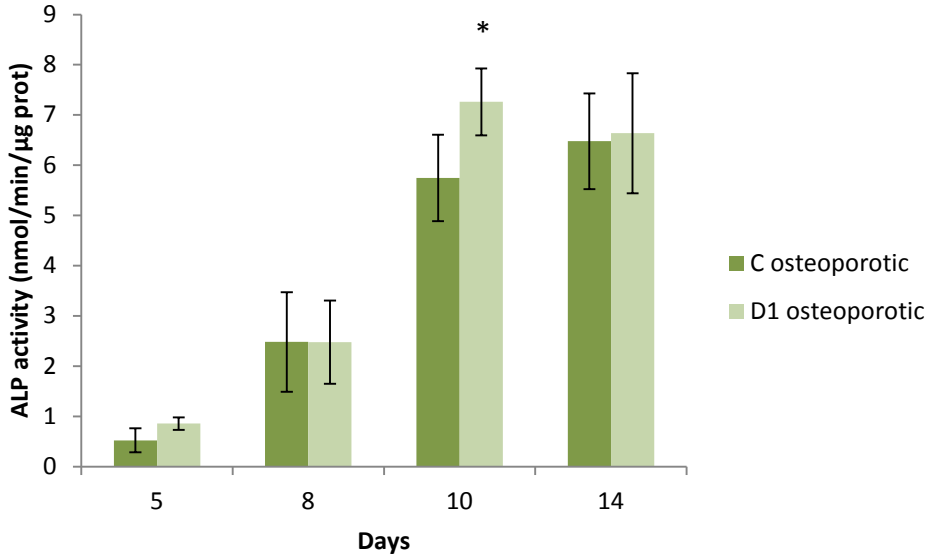


Figure 20 – CLSM imaging of osteoporotic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
Cytoskeleton was stained in green and nucleus counterstained in red. A to C corresponds to control cultures, and D to F corresponds to cultures with doxycycline. Magnification 400x.

ALKALINE PHOSPHATASE ACTIVITY

Results of ALP activity were normalized by total protein content and are show in Graphic 16. In the control osteoporotic culture, ALP activity increased over the time of culture. The cultures established in the presence of doxycycline demonstrated a similar time-course growth pattern – increased ALP activity values from the first day till the last day of culture. Doxycycline culture showed higher ALP activity than the control culture during the entire culture time.



Graphic 16 – ALP activity of osteoporotic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
* – significantly different from control ($p < 0.05$).

ALKALINE PHOSPHATASE STAINIG

Images shown in Figure 21 are representative of ALP staining. At day 5 clusters of cells can be acknowledged, which start to grow and cover a vast majority of the culture plate in later time points, days 10 and 14. The staining intensity also increased over the time of culture, passing through a light brown in day 5 to a dark brown/black in days 10 and 14. There are no significant differences between the two experimental conditions.

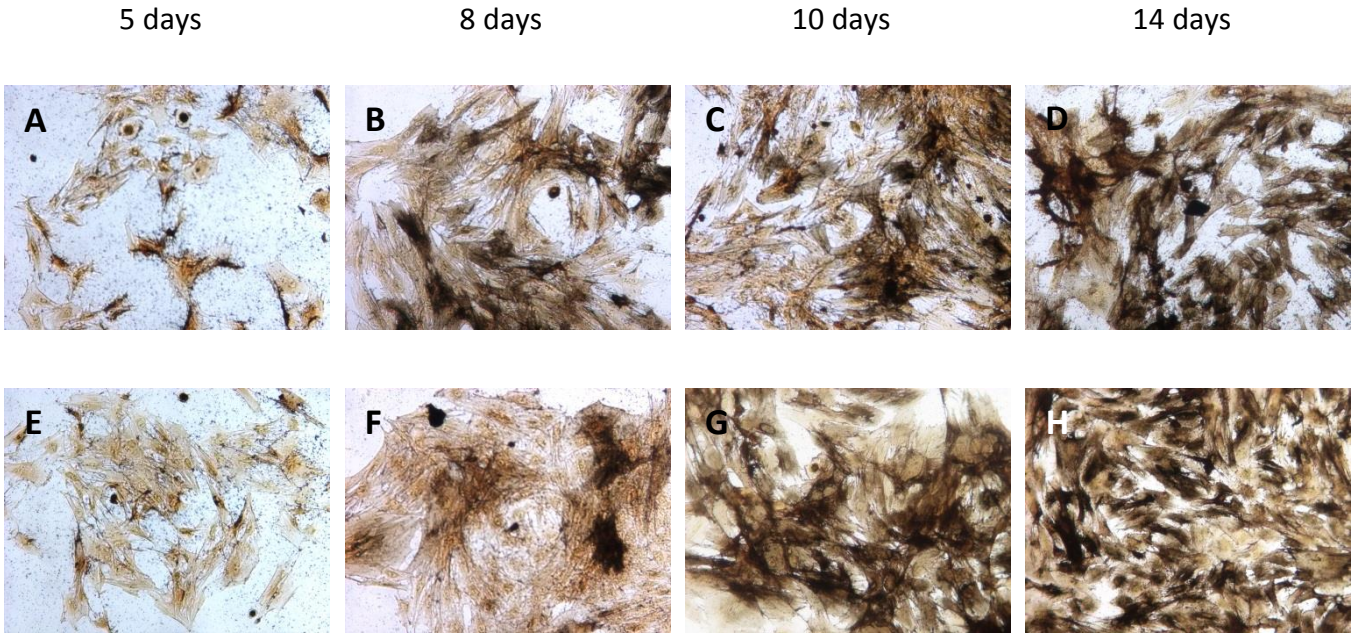


Figure 21 – ALP staining of osteoporotic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
A to D corresponds to control cultures, and E to H corresponds to cultures with doxycycline. Magnification 100x.

COLLAGEN STAINING

Figure 22 is representative of collagen staining in osteoporotic cultures with and without doxycycline. At day 5 it's visible the presence of nodular aggregates that increased and become more intense. The staining at day 5 is very light and it becomes darker from day 8 to day 10. At days 10 and 14 the pink is more intensive and the cell structure occupy almost the whole culture plate. The staining and organization pattern are similar in both experimental conditions.

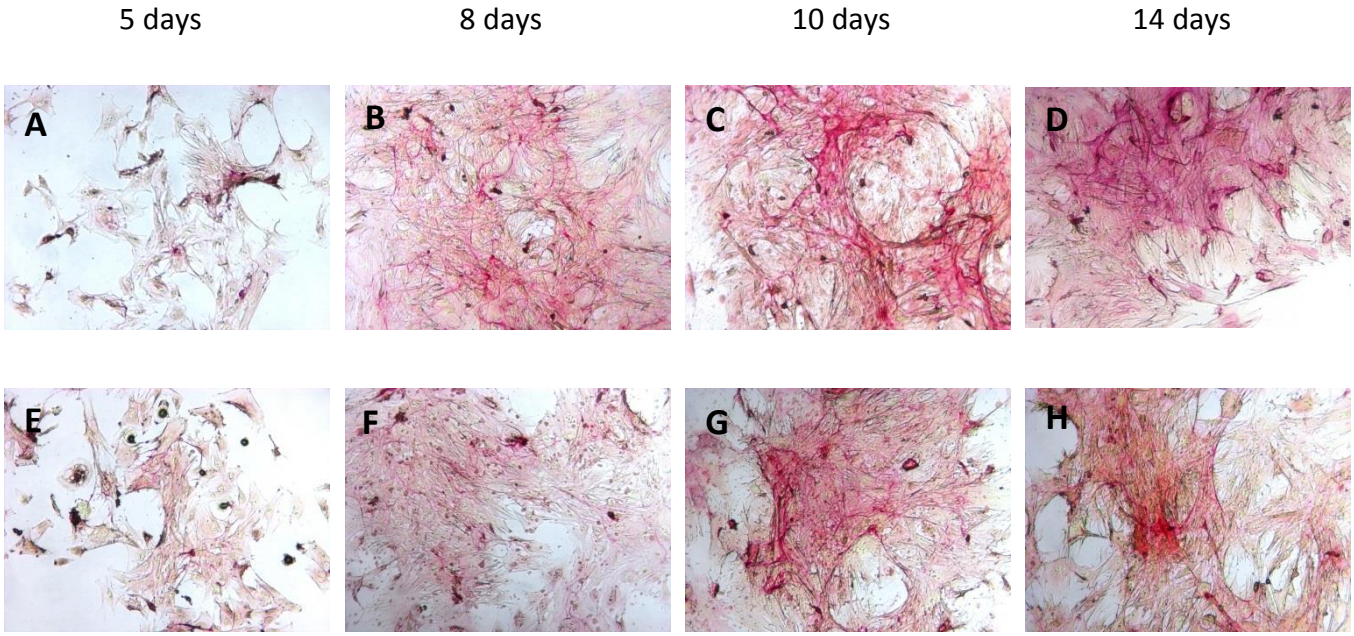
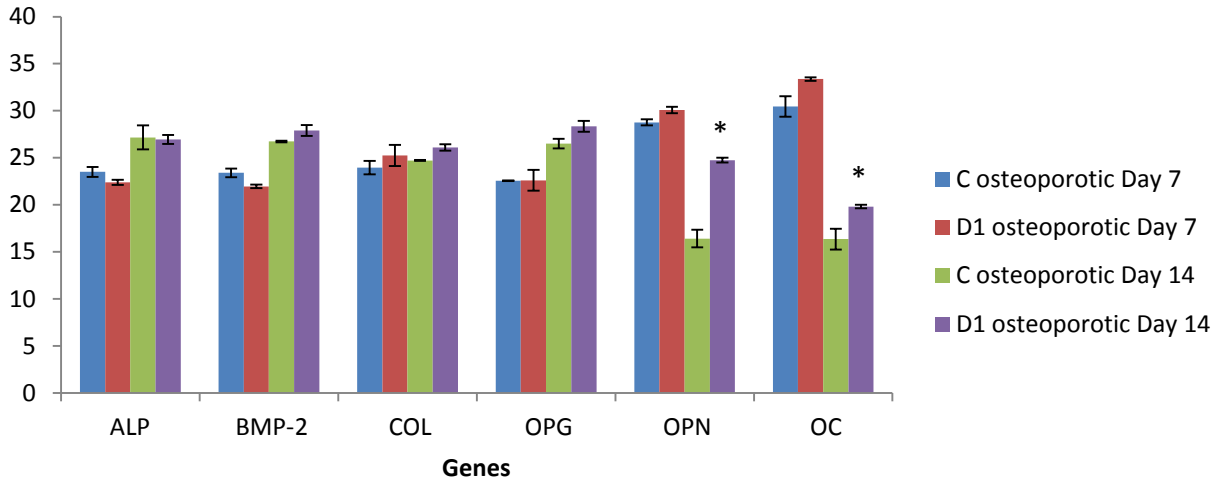


Figure 22 – Collagen staining of osteoporotic rat bone marrow-derived cell cultures, established for 14 days, in the absence and in the presence of doxycycline.
A to D corresponds to control cultures, and E to H corresponds to cultures with doxycycline. Magnification 100x.

EXPRESSION OF OSTEOGENIC-RELATED MARKERS

RT-PCR analysis showed that both experimental conditions – osteoporotic control cultures and with doxycycline – expressed high levels of significant osteogenic markers. Results are shown in Graphic 17.

At day 7, cultures with doxycycline expressed higher levels of Col I, OPG, OPN and OC than the osteoporotic control culture. On the other hand, at the same time point, cultures with doxycycline expressed reduced levels of ALP and BMP-2 in comparison with control cultures. At day 14, the expression of osteogenic markers increased with addition of doxycycline.



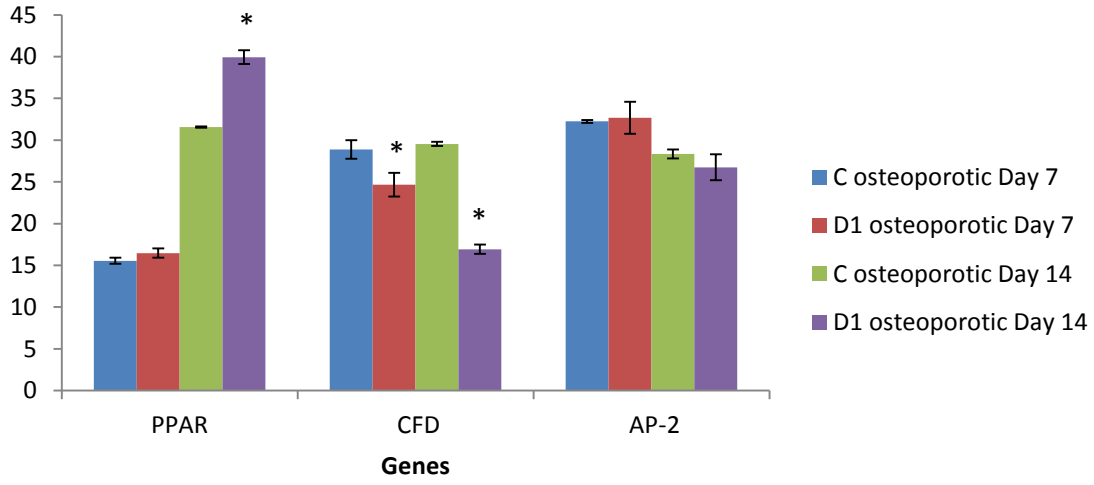
Graphic 17 – RT-PCR gene expression of ALP, BMP-2, Col I, OPG, OPN and OC, in osteoporotic rat bone marrow-derived cell cultures established at days 7 and 14, in the absence and in the presence of doxycycline.

* – significantly different from control ($p < 0.05$).

EXPRESSION OF ADIPOGENIC-RELATED MARKERS

RT-PCR analysis of adipogenic markers is shown in Graphic 18.

At day 7, the expression levels of CFD decreased in cultures with doxycycline. At day 14, the expression of PPARγ increased, while the CFD expression decreased, in comparison with the control culture.



Graphic 18 – RT-PCR gene expression of PPARγ, CFD and AP-2, in osteoporotic rat bone marrow-derived cell cultures established at days 7 and 14, in the absence and in the presence of doxycycline.

* – significantly different from control ($p < 0.05$).

V. DISCUSSION

Osteoporosis is a condition very current and increasingly common in the world population, and thus a matter of high concern and interest. There is a high predisposition to fractures that cause pain and disability and their occurrence is more common in women (4). An imbalance in the processes of bone formation and bone resorption may be the cause of this disease (49). Osteoporosis can be characterized by loss of bone mass and changes in microscopic architecture of the bone. As a result, bone becomes more brittle and fracture risk increases (31,32). It is known that bone has the capacity of self-regeneration; however, bone regeneration is a complex process not yet completely understood, especially when osteoporosis is present.

Numerous investigations have addressed the bone healing and regeneration processes using animal models of osteoporosis. Rodent models have an extensive employment due to their availability, easy of manipulation, relative low cost and biological significance.

In this work two animal models were established, the osteopenic and the osteoporotic rat. Osteopenia was established following the physiological development of the aging process. Animals with 48 weeks were used within this group. Microtomographic evaluation the proximal tibia revealed a discrete, yet significant reduction of the BV/TV, Tb N and Mean Density, supporting the state of osteopenia. Furthermore, osteoporosis was established with an ovariectomy surgical procedure and induced fast modifications within bone morphometric parameters. OVX animals, euthanized at 20 weeks of age, revealed a reduction in the BV/TV and Tb N of more than around 3 times. A significant reduction of the Mean Density was also attained. Verified differences, due to attained magnitude, corroborate the establishment of a model of the osteoporotic condition. In order to compare the developed models with a physiological control, sham animals – in which a sham ovariectomy procedure was conducted – were euthanized at 20 weeks of age.

Following the establishment of the animal models, the *in vitro* protocol was divided in three parts, in which three metabolic conditions were evaluated. To do that, we established bone marrow-derived osteoblastic cultures from sham, osteopenic and osteoporotic animals, at the reported time points.

A comparative approach allowed to identify some patterns in the cellular behavior of the cultures derived from the distinct metabolic conditions. Briefly, in which regards cell proliferation, the cultures established from osteopenic animals demonstrated a slower proliferation and the lowest values of DNA. The metabolic activity revealed a similar pattern in the three cultures, which reached the confluence at day 10 and decreased at day 14. However,

the cultures from osteopenic animals were the ones with lower MTT values during the culture time. Sham cultures were the ones that started with higher MTT values, having a less accentuated increase, while osteoporotic cultures started with lower values but reached similar ones of those obtained in the sham culture at day 10. In terms of functional activity, osteopenic cultures were the ones with lower ALP activity, followed by osteoporotic and sham cultures, which presented the higher ALP enzymatic activity.

These results come in line with previous established research. A large body of literature indicates that age-related impaired bone formation is the principal pathogenic mechanism mediating age-related bone loss (90). Our model of osteopenia is essentially age-dependent and reported evidence converges to assist that the impaired bone formation results from age-related decreased in osteoblast number and function, as consequences of multitude intrinsic senescence-related mechanisms (90). Essential mechanisms include an impairment of the cell proliferation, decline in the cellular lifespan, and the impairment of the differentiation, function, and response capability to endogenous and exogenous factors (91-93). Furthermore, genetic-related factors also seem to be of importance, and can be grouped into two defined groups: damage-related factors and maintenance (repair) factors. Disturbances in the balance between damage and maintenance factors increase the risk of cellular senescence and dysfunction (90).

Furthermore, in the bone marrow microenvironment, both fat and bone tissue co-exist and several histomorphometric studies have demonstrated that the observed decrease in trabecular bone volume is correlated with an increased bone marrow adipocyte tissue volume, during the aging process. This consideration, described as ‘the inverse relationship between adipocyte and osteoblast differentiation’, was supported by experimental evidence in MSC cultures of animal models (94). More recent human studies revealed that the adipocyte-forming capacity of MSC does not change with age and that no age-related change in the expression of mRNA levels of adipocyte and osteoblast differentiation markers was detected in humans (95). These later results come in line with the gene expression results, in which a similar level of expression of both osteogenic and adipogenic markers was verified in both sham and osteopenic-derived cultures.

In which regards to the osteoporotic condition, the loss of bone mineral density is attributable to a pathological imbalance between bone resorption and bone formation during the remodeling process. Whereas the postmenopausal osteoporosis is mainly attributable to the increased bone resorbing activity of osteoclasts caused by estrogen deficiency, senile osteoporosis is attributed to inadequate osteoblastic function (96). A large number of experimental studies indicate that, in osteoporotic conditions, osteoblasts are characterized by

lower proliferation and defective function compared with normal osteoblasts. Related mechanisms might involve abnormalities of the IGF-I signaling system (97), a different production pattern of cytokines involved in the regulation of bone metabolism (98), which converge to occurrence of a different metabolic phenotype in osteoporotic osteoblasts and indicate the presence of reduced anabolic function (99). Furthermore, animal models and *in vitro* studies have demonstrated a disequilibrium in the RANK/RANKL/OPG system in osteoporotic conditions. A human *in vitro* study has further suggested that the up-regulation of RANKL on bone marrow cells is an important determinant of increased bone resorption induced by estrogen deficiency (55).

Overall, within the established *in vitro* model of osteoblastic cultures derived from three distinct metabolic conditions, it was verified that the aging-related osteopenic condition was found to greater affect the functionality of cultured cells, sustaining a preponderant effect of the senescence process over the ovariectomy – osteoporotic condition. Despite this realization, microtomographic indexes were found to be more significantly affected in osteoporotic animals sustaining that the metabolic function/functional activity of bone marrow-derived osteoblasts might not be directly related to morphometric indexes of the bone tissue.

Besides that, we aimed to assess the doxycycline effect in these three conditions. Tetracyclines are a well-characterized family of antibiotics that may have specific therapeutic value in the treatment of bone diseases, since they have great affinity for mineralized bone matrix (100). Besides that, they have been found to exert an extensive variety of catabolic actions over the metabolic balance of the bone tissue by modulating both osteoblastic and osteoclastic functions (101). Doxycycline is a representative of broad-spectrum tetracyclines antibiotics that is extensively used in medical and dental practice. These antimicrobial drugs revealed an overall positive effect on bone, being considered potential inductive agents for the management of the osteopenic and osteoporotic conditions (102).

Knowing the ability of tetracyclines to improve the osteogenic function in a wide range of models, both in physiological and pathological conditions (like osteoporosis), the choice of using it was based on the possibility of enhancing the osteogenic potential of osteoblast precursor cells in the metabolic conditions above mentioned – sham, osteopenia and osteoporosis. Therefore, when accomplished the subculture, doxycycline (1 µg/ml) was added to the established cultures, in the absence of any osteogenic inducer.

In the first part of this study we established and characterized bone marrow derived osteoblastic cultures from sham animals. Were settled two conditions – control and with doxycycline at 1 μg/ml. The cells were then characterized assessing the cell proliferation, metabolic and functional activities. Cell proliferation was evaluated by the DNA content and the results showed an increased over the time of culture. The addition of doxycycline showed to induce the proliferation of cells. Metabolic activity, assessed by the MTT assay, increased in the first 10 days of culture and decreased at day 14, in the control cell culture. Culture with doxycycline presented a similar time-course growth pattern – metabolic activity increased till day 10, day where the confluence was reached, and decreased at fourteenth day. Comparing the two cultures, it's visible that doxycycline stimulated metabolic activity in the first 8 days of culture, but from day 10 to day 14 the control culture showed higher MTT reduction values. A similar behavior is found in functional activity – ALP activity increased till day 10 and diminished at day 14. This activity is stimulated by doxycycline at days 8 and 10. Histochemical staining for ALP correlated the biochemical determination, revealing a high and increased stain intensity and nodular aggregation throughout the time of culture. The histochemical for collagen also showed an increase in staining in both cultures. The apoptosis, assessed by the Caspase-3 activity, was constant during the time of culture and it was not altered with the introduction of doxycycline.

The RT-PCR analysis of osteogenic markers revealed that the expression level of ALP, BMP-2, Col I, OPG and OPN was similar at day 7 and the addition of doxycycline stimulated the expression of these markers. At day 14, the expression level of ALP, BMP-2, Col I and OPG was almost the same, but OPN and OC were expressed in reduced levels. As expected, doxycycline addition enhanced the expression of ALP, such as the expression of OPN and OC. However, the expression levels of BMP-2, Col I and OPG slightly decreased.

In terms of expression of adipogenic markers, at day 7, PPARγ and AP-2 were expressed at a similar level and the addition of doxycycline decreased their expression. CFD was expressed in higher levels at day 7 and its expression was enhanced with introduction of doxycycline. At day 14, PPARγ and AP-2 were the markers with higher levels of expression. However, at this time point, the addition of doxycycline reduced the expression of all the adipogenic markers.

In the second part of this study, the aim was to establish and characterize bone marrow-derived osteoblastic cultures from osteopenic animals. Once again, were stated two conditions – control and with doxycycline to enhance osteogenic function – and cell proliferation, metabolic and functional activities were then assessed. Cell proliferation increased throughout the time of culture and it's evident that the presence of doxycycline stimulated cell proliferation, especially in the last days of culture. In metabolic activity the cultures presented a different behavior, in both cultures the metabolic activity increased till day 10, day where confluence was reached, and decreased at day 14. However, the culture with doxycycline showed lower MTT values than the control culture. In terms of Caspase-3 activity, the apoptosis had a decline at day 10 but returned to normal values at day 14. There were no significant differences between the two experimental conditions. The ALP activity showed a similar behavior in both conditions, increasing over the time of culture. The addition of doxycycline enhanced the ALP activity in the eighth and tenth days of culture. These results were corroborated with histochemical data, in which it's possible to see the increasing in nodular aggregates and stain intensity. Being ALP an important osteoblastic marker, the osteoblastic phenotype was maintained in the two conditions. Collagen staining revealed an increasing in the coloration with no evident differences between the two cultures.

ALP is one of the most important genetic markers for osteoblasts being expressed during osteoblastic differentiation. The expression of ALP showed a slight increase from day 7 to day 14 and, in both time points, it was intensified with the addiction of doxycycline. OPN, other marker for osteoblast differentiation, showed the higher levels of expression at day 7 in both conditions; however, this marker showed the lower expression levels in the control culture at day 14 and was enhanced with doxycycline. The expression of BMP-2, Col I and OPG didn't show significant differences in the two conditions at day 5 but the expression of these markers increased at day 14 and it was stimulate with introduction of doxycycline. The expression of OC, often used as a marker for the bone formation process, didn't show significant differences at day 5 in both conditions, but the addiction of doxycycline stimulated greatly its expression at day 14. In general, the expression of osteogenic markers increased with addition of doxycycline, being this other parameter that confirms that this antibiotic stimulates the differentiation of osteoblasts.

In terms of the expression of adipogenic markers, PPARγ, a marker expressed in adipocytes, showed reduced levels of expression at day 7 that increased at day 14 and, in both time points, its expression levels were enhanced by addition of doxycycline. CFD was the adipogenic marker that revealed a higher expression at day 5 being reduced with doxycycline. At day 14, the expression of CFD in control cultures was lower than at day 14 but it was stimulated when doxycycline was introduced. AP-2 expression increased with introduction of doxycycline in both experimental conditions. Despite the different levels of adipogenic expression, doxycycline stimulated the expression of all of the genes used. In terms of values, the levels of expression of adipogenic markers are similar to those obtained in osteogenic markers, so the established osteopenic culture had a propensity to the adipogenic differentiation alike the osteogenic one.

In the third part of this work, we had the goal of establish and characterize bone marrow-derived osteoblastic cultures from osteoporotic animals. The osteoblastic cells were cultured with and without doxycycline and cell proliferation, metabolic and functional activities were evaluated. Cell proliferation of the control culture increased from day 5 to day 10 and decreased at day 14. On tenth day of culture, control cells cultures reached confluence, and cell proliferation is inhibited by cell to cell contact what justify the decrease at the fourteenth day. Culture with doxycycline didn't present this type of behavior, the proliferation rate increased throughout the whole time of culture, so doxycycline presented a slower cell proliferation rate and its peak was reached later, at day 14. Metabolic activity of control culture increased till day 10 of culture and decreased at day 14, while culture with doxycycline showed an increase over the 14 days of culture. Accordingly, doxycycline delays the metabolic activity and the cells achieve confluence later. There were no substantial differences between the two cultures in apoptosis, being the apoptosis values low in both experimental conditions, so doxycycline doesn't interfere in the controlled cell death. In terms of functional activity, control culture revealed a regular increase in ALP throughout the time culture. The addition of doxycycline showed a stimulatory effect, increasing the activity of ALP since the first day of culture, being this outcome more marked at day 10. Histochemical results for ALP underpinned the biochemical data being visible the increase in staining intensity and number of clusters in both conditions. At day 10 it was also detectable a darker coloration in the doxycycline culture when compared with the control. The histochemical for collagen revealed an increasing in staining intensity for both cultures and no significant differences were visible.

In terms of the gene expression of osteogenic markers, ALP and BMP-2 had a similar level of expression at day 7 and then suffered a slight decrease when doxycycline was added. At day 14, the expression of both markers increased but, while ALP revealed a decrease in the culture with doxycycline, the expression of BMP-2 was stimulated by doxycycline. Weren't found significant differences in Col I expression at days 7 and 14, and it's evident that doxycycline enhanced its expression in both time points. The expression level of OPG at day 7 was similar in both experimental conditions. At day 14, the OPG expression increased which was stimulated with addition of doxycycline. This pattern of expression wasn't observable in OPN and OC expression; these two genes showed the highest level of expression at day 7 that was stimulated when doxycycline was introduced. However, at day 14 their expression was much reduced, but doxycycline revealed a stimulatory effect once again.

The expression of adipogenic markers was really irregular and showed different results. At day 7 the expression levels of PPARγ and AP-2 were low, while CFD was expressed in elevated levels. The doxycycline had different effects in these markers, stimulating the expression of PPARγ and AP-2 and inhibiting the CFD expression. At day 14 the osteoporotic cells from the control culture expressed PPARγ, CFD and AP-2 at a similar level; however, the doxycycline enhanced the expression of the first one and decreased the expression of the other two markers. With these results we can see that the established osteoporotic culture didn't have great propensity to differentiate into an adipogenic phonotype at day 7, but at day 14 the levels of expression were higher, so it's possible that the adipogenic differentiation happens later in the culture time.

The results obtained in this experimental work are in line with the evidences found in the literature about bone metabolism and pathology in animal models. In a mouse model used to simulate diabetic-induced osteopenia was verified that the pathological situation (characterized by decreased bone formation and not augmented bone resorption) was significantly ameliorated with tetracycline uptake (103). Bone regeneration in a dog model revealed that the administration of tetracycline induced a more regenerative healing and minimized crestal resorption, in comparison to control (104). Also in a dog model, the administration of doxycycline reduced the severity degree of osteoarthritis, with reduced levels of total collagenase activity and inhibition of the proliferation and hypertrophy of chondrocytes (105). These results were later confirmed in a double-blinded, randomized, placebo-controlled trial that reported a doxycycline-dependent reduction in the rate of joint space narrowing, in knees of obese women with established osteoarthritis (106). Studies with ovariectomized rats revealed that low dose of tetracycline administration increased bone

formation and mass levels (107). Tetracycline administration to squirrel monkeys, in a model of normal bone metabolism, increased the deposition of osteoid in the alveolar process by increasing the number of active osteoblasts (108).

In human bone marrow-derived osteoblastic cells, doxycycline has increased significantly the number of active osteoblastic cells that provided a proportional amount of a normal mineralized ECM (109). This behavior was further confirmed when the human osteoblastic cultures were grown on the surface of two biomaterials for bone regeneration – HA and a glass-ceramic composite (110). A similar effect was found in human osteoblasts, in which doxycycline enhanced cell maturation and differentiation rather the process of proliferation itself (111). Recently, studies revealed that doxycycline at 1 μg/ml increase the OC, osteonectin and ALP expression in human periodontal ligament cells. Besides that, the increase in ALP expression was found to be more significant than the one induced by BMP-2 (112).

Regarding to bone metabolism relevance, tetracyclines showed to have some effect in the modulation of the osteoclastogenic response. Recent studies found that doxycycline and minocycline (is also a broad-spectrum tetracyclines antibiotics) have the ability to inhibit the RANKL-induced osteoclastogenesis of precursor cells, but they didn't show any effects on cell growth and phagocytic activity. In addition, the authors also reported the absence of effect on cell proliferation or differentiation in bone-forming osteoblast. Similarly, were perfomed some *in vivo* studies which revealed that the injection of tetracyclines into RANKL-injected mice and RANKL-transgenic mice suppressed the RANKL-mediated osteoclastogenesis process and promoted the concomitant appearance of $CD11c^{+}$ cells (113).

VI. CONCLUSION

Osteoporosis is a prevalent disease characterized by a low bone mass and changes in the microarchitecture of the bone tissue, which result in increased bone fragility and fracture risk. In this pathological condition, the new bone formation decreases and the bone regeneration process can be affected. This way, bone tissue engineering strategies require biomodulators to achieve a successful regeneration of the bone tissue and tetracyclines are a good example of this type of biomodulators.

In this work, there were established bone marrow-derived cultures from sham, osteopenic and osteoporotic animals and it was assessed the effect of tetracycline in these three conditions. Cell cultures established from the sham group shown an increased cell proliferation in the presence of doxycycline. Doxycycline was also found to enhance metabolic activity and ALP expression of the established cultures. Doxycycline stimulated cell proliferation and ALP activity in cultures established from osteopenic animals. This stimulatory effect of doxycycline was corroborated by the high expression of osteogenic markers such as ALP, BMP-2, Col I, OPG, OPN and OC. In cultures established from osteoporotic animals, doxycycline showed a delay in cell proliferation and in metabolic activity; however, the expression of ALP was stimulated, what confirmed the maintenance of an osteoblastic cell-like phenotype. RT-PCR evaluation of significant osteogenic markers also revealed an enhancement with addition of doxycycline.

The results presented in this study showed that doxycycline can be a suitable candidate to improve the osteogenic potential of osteoblast precursor cells in osteopenic and osteoporotic conditions. Therefore, these antimicrobial drugs can be useful in tissue engineering and have a potential biomedical application, being used in the treatment of osteoporosis

VII. REFERENCES

1. Buckwalter J, Cooper R. Bone structure and function. Instr Course Lect. 1987: p. 27-48.

2. Manolagas S. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev. 2000; 21(2): p. 115-37.

3. Seeley R, Stephens T, Tate P. Sistema Esquelético: Ossos e Tecido Ósseo. In Anatomia e Fisiologia. 6th ed.: Lusociência; 2003. p. 173-204.

4. Seeman E. Invited Review: Pathogenesis of osteoporosis. J Appl Physiol. 2003; 95: p. 2142–2151.

5. Riancho J, Delgado-Calle J. Osteoblast-osteoclast interaction mechanisms. Reumatol Clin. 2011.

6. Parfitt A. Targeted and Nontargeted Bone Remodeling: Relationship to Basic Multicellular Unit Origination and Progression. Bone. 2005; 30(1): p. 5-7.

7. Mackiewicz Z, Niklińska WE, Kowalewska J, Chyczewski L. Bone as a source of organism vitality and regeneration. Folia Histochem Cytobiol. 2011; 49(4): p. 558–569.

8. David Wooster Middle School. [Online].; 2008 [cited 2012 Junho 28. Available from: http://woostermiddle.stratfordk12.org/.

9. Sistema Esquelético. [Online]. [cited 2012 Junho 19. Available from: http://www.auladeanatomia.com/osteologia/generalidades.htm.

10. Khosla S, Westendorf J, Oursler MJ. Building bone to reverse osteoporosis and repair fractures. J Clin Invest. 2008; 118: p. 421–428.

11. Hillendale Health. [Online]. [cited 2012 Julho 1. Available from: http://hes.ucfsd.org/gclaypo/skelweb/skel01.html.

12. Chapter 6: Bones And Skeletal Tissues. [Online]. [cited 2012 Junho 19. Available from: http://classes.midlandstech.edu/carterp/Courses/bio210/chap06/lecture1.html.

13. Griffin JE, Ojeda SR. Calcium Homeostasis. In Textbook of Endocrine Physiology. 5th ed. New York: Oxford University Press, Inc; 2004. p. 349-377.

14. Institute BaNC. Campact Bone & Spongy (Cancellous Bone). In The Encyclopedia of Science.

15. Vaes LB, Dechering KJ, Feijen A, Hendriks JM, Lefèvre C, Mummery CL, et al. Comprehensive Microarray Analysis of Bone Morphogenetic Protein 2–Induced Osteoblast Differentiation Resulting in the Identification of Novel Markers for Bone Development. Journal of Bone and Mineral Research. 2002.

16. Lian J, Stein G. Development of the osteoblast phenotype: molecular mechanisms mediating osteoblast growth and differentiation. Iowa Orthop J. 1995: p. 118–140.

17. Clines G. Prospects for Osteoprogenitor Stem Cells in Fracture Repair and Osteoporosis. Curr Opin Organ Transplant. 2010; 15(1): p. 73–78.

18. Gilbert SF. In Developmental Biology. 6th ed.; 2000.

19. Cummings B. In.: Pearson Education; 2006.

20. Rodan G, Martin T. Role of osteoblasts in hormonal control of bone resorption—A hypothesis. Calcified Tissue International. 1981: p. 349-351.

21. Ben Azouna N, Jenhani F, Regaya Z, Berraeir L, Ben Othman T, Ducrocq E, et al. Phenotypical and functional characteristics of mesenchymal stem cells from bone marrow: comparison of culture using different media supplemented with human platelet lysate or fetal bovine serum. Stem Cell Res Ther. 2012; 3(1): p. 6.

22. Oursler MJ, Bellido T. ASBMR. [Online].; 2003 [cited 2012 Junho 19. Available from: http://depts.washington.edu/bonebio/ASBMRed/cells.html.

23. Marquis ME, Lord E, Bergeron E, Drevelle O, Park H, Cabana F, et al. Bone cells-biomaterials interactions. Frontiers in Bioscience. 2009; 14: p. 1023-1067.

24. Bonewald L. The Amazing Osteocyte. Journal of Bone and Mineral Research. 2011; 26(2): p. 229–238.

25. Everts V, Delaissé J, Korper W, Jansen D, Tigchelaar-Gutter W, Saftig P, et al. The Bone Lining Cell: Its Role in Cleaning Howship's Lacunae and Initiating Bone Formation. J Bone Miner Res. 2002; 17(1): p. 77–90.

26. Karsenty G. The complexities of skeletal biology. Nature. 2003; 423.

27. Neve A, Corrado A, Cantatore FP. Osteoblast physiology in normal and pathological conditions. Cell Tissue Res. 2011; 343: p. 289-302.

28. Baynes J, Dominiczak M. Calcium and Bone Metabolism. In Medical Biochemistry. Philadelphia: Elsevier Mosby; 2005. p. 345-358.

29. Niewoehner CB. Calcium-Regulating Hormones and Metabolic Bone Disease. In Endocrine Pathophysiology. 2nd ed. North Carolina: Hayes Barton Press; 2004. p. 124-163.

30. Hall J, Saunders. In Guyton and Hall Textbook of Medical Physiology. 12th ed.; 2010.

31. Carbonare LD, Valenti MT, Zanatta M, Donatelli L, Lo Cascio V. Circulating Mesenchymal Stem Cells With Abnormal Osteogenic Differentiation in Patients With Osteoporosis.

Arthritis & Rheumatism. 2009; 60(11): p. 356–3365.

32. Erdogan Ö, Shafer D, Taxel P, Freilich M. A review of the association between osteoporosis and alveolar ridge augmentation. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007; 104: p. 738.e1-738.e13.

33. Kwak E, Lee Y, Choi E. Effect of magnolol on the function of osteoblastic MC3T3-E1 cells. Mediators Inflamm. 2012.

34. Pierre M, Kassem M. Osteoblasts in osteoporosis: past, emerging, and future anabolic targets. European Journal of Endocrinology. 2011; 165: p. 1-10.

35. Hernández-Gil IFT, Gracia MAA, Pingarrón MdC, Jerez LB. Physiological bases of bone regeneration II. The remodeling process. Med Oral Patol Oral Cir Bucal. 2006;: p. E151-157.

36. [Online]. [cited 2012 Maio 20. Available from: http://www.ns.umich.edu/Releases/2005/Feb05/bone.html.

37. Cummings SR, Cosman F, Jamal SA. Bone Biology, Epidemiology, and General Principles. In Osteoporosis: An Evidence-Based Guide to Prevention and Management. Philadelphia: American College of Physicians-American Society of Internal Medicine; 2002. p. 3-29.

38. Draznin B, Epstein S, Turner HE, Wass JA. Calcium and bone metabolism. In Oxford American Handbook of Endocrinology and Diabetes. New York: Oxford University Press Inc; 2011. p. 365-429.

39. Garcia-Gomez A, Ocio E, Crusoe E, Santamaria C, Hernández-Campo P, Blanco J, et al. Dasatinib as a bone-modifying agent: anabolic and anti-resorptive effects. PLoS One. 2012; 7(4): p. e34914.

40. Bilezikian JP, Raisz LG, Rodan G. Vitamin D Gene Regulation. In Principles of Bone Biology. 2nd ed. California: Academic Press; 2002. p. 573-587.

41. Bilezikian JP, Raisz LG, Rodan GA. Structure and Molecular Biology of the Calcitonin Receptor. In Principles of Bone Biology. 2nd ed. California: Academic Press; 2002. p. 603-619.

42. Braddock M, Houston P, Campbell C, Ashcroft P. Born again bone: tissue engineering for bone repair. News Physiol Sci. 2001; 16: p. 886-1714.

43. Langer R. Tissue Engineering. Molecular Therapy. 2000; 1: p. 12-15.

44. Martins A, Alves C, Kasper K, Mikos A, Reis R. Responsive andin situ-forming chitosan scaffolds for bone tissue engineering applications: an overview of the last decade. Journal of Materials Chemistry. 2010; 20: p. 1638-1645.

45. Pollock R, Alcelik I, Bhatia C, Chuter G, Lingutla K, Budithi C, et al. Donor site morbidity following iliac crest bone harvesting for cervical fusion: a comparison between minimally invasive and open techniques. Eur Spine J. 2008; 17: p. 845-852.

46. Davis H, Leach J. Hybrid and composite biomaterials in Tissue Engineering. In Davis H, Leach J. Hybrid and composite biomaterials in Tissue Engineering.: Ed. N Ashammakhi; 2008.

47. Rachner T, Khosla S, Hofbauer L. Osteoporosis: now and the future. Lancet. 2011; 377(9773): p. 1276-87.

48. Pirro M, Leli C, Fabbriciani G, Manfredelli M, Callarelli L, Bagaglia F, et al. Association between circulating osteoprogenitor cell numbers and bone mineral density in postmenopausal osteoporosis. Osteoporos Int. 2010; 21: p. 297–306.

49. Rodríguez JP, Garat S, Gajardo H, Pino AM, Seitz G. Abnormal Osteogenesis in Osteoporotic Patients Is Reflected by Altered Mesenchymal Stem Cells Dynamics. Journal of Cellular Biochemistry. 1999; 75: p. 414–423.

50. University of Maryland - Medical Center. [Online]. [cited 2012 Junho 28. Available from: http://www.umm.edu/imagepages/17156.htm.

51. Windahl S, Vidal O, Andersson G, Gustafsson J, Ohlsson C. Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERβ–/– mice. J Clin Invest. 1999: p. 895–901.

52. Rosen CJ. Skeletal Physiology and its Relevance to Osteoporosis. In Osteoporosis: Diagnostic and Therapeutic Principles. New Jersey: Humana Press; 1996. p. 3-47.

53. Osteoporose - Cuidado para a Saúde. [Online]. [cited 2012 Abril 8. Available from: http://www.osteoprotecao.com.br.

54. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, et al. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterialcalcification. Genes Dev. 1998; 12(9): p. 1260-1268.

55. Eghbali-Fatourechi G, Khosla S, Sanyal A, Boyle W, Lacey D, Riggs B. Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. J Clint Invest. 2003; 111(8): p. 1221-1230.

56. Gourlay M, Fine J, Preisser J, May R, Li C. Bone-density testing interval and transition to osteoporosis in older women. N Engl J Med. 2012; 19: p. 225-233.

57. Fini M, Giavaresi G, Torricelli P, Borsari V, Giardino R, Nicolini A, et al. Osteoporosis and biomaterial osteointegration. Biomedicine & Pharmacotherapy. 2004; 58: p. 487-493.

58. Bandeira F, Carvalho EF. Prevalência de osteoporose e fraturas vertebrais em mulheres na pós-menopausa atendidas em serviços de referência. Rev bras epidemiol. 2007; 10(1): p. 86-89.

59. Osteoporose. [Online]. [cited 2012 Junho 20. Available from: http://osteoporose-info.blogspot.pt/2012/05/epidemiologia-e-aspectos-economicos-da.html.

60. Turner S. Animal Models of Osteoporosis - Necessity and Limitations. European Cells and Materials. 2001; 1: p. 66-81.

61. Stevenson JC, Banks LM, Spinks TJ, Freemantle C, Macintyre l, Hesp R, et al. Regional and Total Skeletal Measurements in the Early Postmenopause. J Clin Invest. 1987; 80: p. 258-262.

62. Stevenson J, Marsh M. An Atlas of Osteoporosis. 3rd ed.: Informa Healthcare; 2007.

63. Ed Kumar V, Abbas A, Aster J, Fausto N. Saunders. Robbins & Cotran Pathologic Basis of Disease. In.; 2009.

64. Orwoll ES, Bliziotes M. Human and Animal Studies of the Genetics of Osteoporosis. In Osteoporosis: Pathophysiology and Clinical Management. New Jersey: Humana Press; 2003. p. 1-33.

65. Stulberg B, Bauer T, Watson J, Richmond B. Bone quality. Roentgenographic versus histologic assessment of hip bone structure. Clin Orthop Relat Res. 1989; 240: p. 200-205.

66. Cummings SR, Cosman F, Jamal SA. Bone Densiometry and Spine Films. In Osteoporosis: An Evidence-Based Guide to Prevention and Management. Philadelphia: American College of Physicians-American Society of Internal Medicine; 2002. p. 29-59.

67. Svendsen OL, Haarbo J, Hassager C, Christiansen C. Accuracy of measurements of body composition by dual-energy x-ray absorptiometry in vivo. Am J Clin Nuir. 1993; 57: p. 605-608.

68. Jergas M, Breitenseher M, Gluer C, Yu W, Genant H. Estimates of volumetric bone density from projectional measurements improve the discriminatory capability of dual X-ray absorptiometry. J Bone Miner Res. 1995; 10(7): p. 1101-1110.

69. Guglielmi G, Grimston S, Fischer K, Pacifici R. Osteoporosis: diagnosis with lateral and posteroanterior dual x-ray absorptiometry compared with quantitative CT. Radiology. 1994; 192(5): p. 845-850.

70. World Health Organization. [Online]. [cited 2012 Junho 20. Available from: http://www.who.int/en/.

71. Brown T, Qaqish R. Antiretroviral therapy and the prevalence of osteopenia and

osteoporosis: a meta-analytic review. AIDS. 2006; 20(17): p. 2165-2174.

72. Sapadin A, Fleischmajer R. Nonantibiotic properties and their clinical implications. Journal of the American Academy of Dermatology. 2006; 54(2)(258-265).

73. Golub L, Ramamurthy N, McNamara T, Grennwald R, Rifkin B. Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. Critical Reviews in Oral Biology & Medicine. 1991; 2(3): p. 297-321.

74. Nelson M. Chemical and biological dynamics of tetracyclines. Adv Dent Res. 1998; 12: p. 5-11.

75. Golub L, Lee H, Lehrer H, G , Nemiroff A, McNamara T, et al. Minocycline reduces gingival collagenolytic activity during diabetes. Journal of Periodontal Research. 1983; 18(5): p. 516-526.

76. Sorsa T, Salo T. Matrix metalloproteinases (MMPs) in oral diseases. Oral Diseases. 2004; 10: p. 311-318.

77. Sasaki T, Ramamurthy N, Golub L. Tetracycline administration increases collagen synthesis in osteoblasts of streptozotoc in-induced diabetic rats: a quantitative autoradiographic study. Calcif Tissue Int. 1992; 50: p. 411-419.

78. Golub L, Ramamurthy N, Llavaneras A, Ryan M, Lee H, Liu Y. A chemically modified nonantimicrobial tetracycline (CMT-8) inhibits gingival matrix metalloproteinases, periodontal breakdown, and extra-oral bone loss in ovariectomized rats. An NY Acad Sci. 1999; 878: p. 290-310.

79. Milch R, Tall D, Tobie J. Bone localization of the tetracyclines. J Natl Cancer Inst. 1957; 19: p. 87-93.

80. Ahler E, Sullivan W, Cass A, Braas D, York A, Bensinger S, et al. Doxycycline alters metabolism and proliferation of human cell lines. PLoS One. 2013; 8(5).

81. Klein N, Cunha B. Tetracyclines. Med Clin North Am. 1995; 79(4): p. 789-801.

82. Boyd S, Muller R, Gasser J. Monitoring individual morphological changes over time in ovariectomized rats by in vivo micro-computed tomography. Bone. 2006; 39: p. 854-862.

83. Hildebrand T, Ruegsegger P. A new method for the model independent assessment of thickness in threedimensional images. J Microsc. 1997; 185: p. 67-75.

84. Ulrich D, van Rietbergen B, Laib A, Ruegsegger P. The ability of three dimensional structural indices to reflect mechanical aspects of trabecular bone. Bone. 1999; 25: p. 55-60.

85. Xuan L, Sasov A. Cluster reconstruction strategies for micro CT and nano Ct scanners. 2005.

86. Berridge M, Tan A. Characterization of the Cellular Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular Localization, Substrate Dependence, and Involvement of Mitochondrial Electron Transport in MTT Reduction. Archives of Biochemistry and Biophysics. 1993: p. 474-482.

87. Twentyman P, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer. 1987: p. 279-285.

88. Hartree E. Determination of protein: A modification of the lowry method that gives a linear photometric response. Analytical Biochemistry. 1972: p. 422-427.

89. Waterborg J, Matthews H. The Lowry Method for Protein Quantitation. In Walker JM, editor. The Protein Protocols Handbook. 2nd ed.: Humana Press; 1996. p. 7-9.

90. Kassem M, Marie P. Senescence-associated intrinsic mechanisms of osteoblast dysfunctions. Aging Cell. 2011; 10: p. 191-197.

91. Zhou S, Greenberger J, Epperly M, Goff J, Adler C, Leboff M, et al. Age-related intrinsic changes in human bone-marrow-derived mesenchymal stem cells and their differentiation to osteoblasts. Aging Cell. 2008; 7: p. 335-343.

92. Fedarko N, Vetter U, Weinstein S, Robey P. Age-related changes in hyaluronan, proteoglycan, collagen, and osteonectin synthesis by human bone cells. J Cell Physiol. 1992; 151: p. 215-227.

93. Karsenty G, Oury F. The central regulation of bone mass, the first link between bone remodeling and energy metabolism. J Clin Endocrinol, Metabol. 2010; 95: p. 4795-4801.

94. Beresford J, Bennett J, Devlin C, Leboy P, Owen M. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. J Cell Sci. 1992; 102(Pt 2): p. 341-351.

95. Justesen J, Stenderup K, Eriksen E, Kassem M. Maintenance of osteoblastic and adipocytic differentiation potential with age and osteoporosis in human marrow stromal cell cultures. Calcif Tissue. 2002; 71: p. 36-44.

96. Beil F, Seitz S, Priemel M, Barvencik F, Von Domuras S, Rueger J, et al. Pathophysiology and pathomorphology of osteoporosis. Eur J Trauma Emerg Surg. 2008; 6: p. 527-534.

97. Perrini S, Natalicchio A, Laviola L, Cignarelli A, Melchiorre M, De Stefano F, et al. Abnormalities of insulin-like growth factor-I signaling and impaired cell proliferation in osteoblasts from subjects with osteoporosis. Endocrinology. 2008; 149: p. 1302-1313.

98. Jurado S, Garcia-Giralt N, Díez-Pérez A, Esbrit P, Yoskovitz G, Agueda L, et al. Effect of IL-1β, PGE2, and TGF-β1 on the expression of OPG and RANKL in normal and osteoporotic primary human osteoblasts. J Cell Biochem. 2010; 110: p. 304-310.

99. Maruotti N, Corrado A, Grano M, Colucci S, Cantatore F. Normal and osteoporotic human osteoblast behaviour after 1, 25- dihydroxy-vitamin D(3) stimulation. Rheumatol Int. 2009; 29: p. 667-672.

100. Eglence A, Colterjohn N, Duivenvoorden WC, Ghert M, Singh G. Effect of bone morphogenetic protein-2 and doxycycline on the differentiation of osteoprogenitors from human femoral bone. The Open Bone Journal. 2009;: p. 1-7.

101. Payne JB, Golub LM. Using tetracyclines to treat osteoporotic/osteopenic bone loss: from the basic science laboratory to the clinic. Pharmacol Res. 2011; 63(2): p. 121-129.

102. Folwarczna J, Pytlik M, Janiec W. Effects of doxycycline on development of changes in histomorphometric parameters of bones induced by bilateral ovariectomy in rats. Pol J Pharmacol. 2005; 55: p. 433-441.

103. Golub L, Ramamthurpy N, Kaneko H, Sasaki T, Rifkin B, McNamara T. Tetracycline administration prevents diabetes-induced osteopenia in the rat: initial observations. Chemical Pathology and Pharmacology. 1990; 68: p. 27.

104. Waleed AA, Bissada N, Greenwell H. The effect of local doxycycline with and without Tricalcium phosphate on the regenerative healing potencial of periodontal osseous defects in dogs. J Periodontol. 1989; 60: p. 582-589.

105. Yu L, Smith G, Brandt K, Myers S, O'Connor B, Brandt B. Reduction of the severity of canine osteoarthritis by prophylactic treatment with oral doxycycline. Arthritis Rheum. 1992; 35: p. 1150-1159.

106. Brandt K, Mazzuca S, Katz B, Lane K, Buckwalter K, Yocum D. Effects of doxycycline on progresion of osteoarthritis. Arthritis & Rheumatism. 2005; 52: p. 2015-2025.

107. Li QN, Hu B, Huang LF, Chen Y, Weng LL, Zheng H. Effects of low doses of hydrochloride tetracycline on bone metabolism and uterus in ovariectomized rats. Acta Pharmacologica Sinica. 2003; 24: p. 599-604.

108. Polson A, Bouwsma O, McNamara T, Golub L. Enhancement of alveolar bone formation after tetracycline administration in squirrel monkeys. The Journal of Applied Research in Clinical Dentistry. 2005;: p. 32-42.

109. Gomes P, Fernandes MH. Effect of therapeutic levels of doxycycline and minocycline in the proliferation and differentiation of human bone marrow osteoblastic cells. Arch Oral Biol. 2007; 52: p. 251-259.

110. Gomes P, Santos J, Fernandes M. Cell-induced response by tetracyclines on human bone marrow colonized hydroxyapatite and Bonelike. Acta Biomater. 2008; 4: p. 630-637.

111. Almazin S, Dziak R, Andreana S, Ciancio S. The effect of doxycycline hyclate, chlorhexidine gluconate, and minocycline hydrochloride on osteoblastic proliferation and differentiation in vitro. J Periodontol. 2009; 80: p. 999-1005.

112. Muthukuru M, Sun J. Doxycycline counteracts Bone Morphogenic Protein-2 induced osteogenic mediators. J Periodontol. 2012.

113. Kinugawa S, Koide M, Kobayashi Y, Mizoguchi T, Ninomiya T, Muto A. Tetracyclines convert the osteoclastic-differentiation pathway of progenitor cells to produce dendritic cell-like cells. J Immunol. 2012; 188: p. 1772-1781.